US006268178B1

(12) **United States Patent**
Kordyum et al.

(10) **Patent No.: US 6,268,178 B1**
(45) **Date of Patent: Jul. 31, 2001**

(54) **PHAGE-DEPENDENT SUPER-PRODUCTION OF BIOLOGICALLY ACTIVE PROTEIN AND PEPTIDES**

(75) Inventors: **Vitaliy A. Kordyum; Svetlana I. Chernykh; Irina Y. Slavchenko; Oleksandr F. Vozianov**, all of Kiev (UA)

(73) Assignee: **Phage Biotechnology Corp.**, Irvine, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/318,288**

(22) Filed: **May 25, 1999**

(51) **Int. Cl.**[7] **C12P 21/00**; C12N 7/00; C12N 1/21; C12N 15/70; C07H 21/04

(52) **U.S. Cl.** **435/69.1**; 435/69.51; 435/320.1; 435/252.3; 435/471; 435/235.1; 536/23.1

(58) **Field of Search** 435/69.1, 252.3, 435/69.4, 69.51, 243, 320.1, 235.1, 471; 536/23.1

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,637,980	* 1/1987	Auerbach et al.	435/68
4,710,463	12/1987	Murray .	
4,716,112	12/1987	Panayotatos .	
4,775,622	10/1988	Hitzeman et al. .	
5,102,797	4/1992	Tucker et al. .	
5,196,318	3/1993	Baldwin et al. .	
5,346,830	9/1994	Christie .	
5,641,673	6/1997	Haseloff et al. .	
5,646,013	7/1997	Takano et al. .	
5,834,209	11/1998	Korsmeyer .	
5,834,233	11/1998	Molin et al. .	
5,861,273	1/1999	Olson et al. .	

FOREIGN PATENT DOCUMENTS

0 140 864	5/1985	(EP) .
0043980	9/1987	(EP) .
0 372 707	6/1990	(EP) .
2130222A	5/1984	(GB) .
2143238A	2/1986	(GB) .

OTHER PUBLICATIONS

Friedman, David and Gottesman, Max. Lytic mode of Lambda development. In Lambda II. Roger Hendrix, ed. pp21–51, Cold Spring Harbor Laboratory Press, 1983.*
Henthorn, et al. Journal of Molecular Biology 257: 9–20, 1996.*
Berendson, et al. Science 282: 642–643, 1998.*
Chen, et al. "Temperature Induction of Bacteriophase λ mutants in *Escherichia coli.*" *Journal of Biotechnology.* 1995. pp. 87–97.
N.E. Murray et al., "Manipulation of restriction targets in phage λ to form receptor chromosomes for DNA fragments", Nature 251, pp. 476–481, 1974.
B. Fischer et al., "Isolation, Renaturation, and formation of disulfide bonds of eukaryotic proteins expressed in *escherichia coli* as inclusioin bodies", P. Biotech and Bioenginerring, pp. 41:3–13, 1993.
T. Mantiatis et al., "Molecular Cloning: A Laboratory Manual", 1982, Cold Spring Harbor Laboratory Press, pp. 17–27.
A. Moir et al., "The use of specialised transducing phages in the amplification of enzyme production", Molec. Gen. Genet., 149, pp. 87–99, 1976.
S.M. Panasenko et al., "Five–Hundredfold overproduction of DNA ligase after induction of a hybrid lambda lysogen constructed in vitro", Science, 196, pp. 188–189, 1977.
N.E. Murray et al., "Characterization of λpolA transducing phages; effective expression of the *E. coli polA*", Molec. Gen. Genet, 175, pp. 77–87, 1979.
S. M. Chaykovakaya, English Abstract Only, Antibiotics 7(5): 453–456, 1962.
J.H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, 1972, Experiment 57, 60 and 62.
Koh et al., "Vectors lambda 200g and lambda 200c: two useful derivatives of lambda 2001", Gene 130, pp. 117–119, 1993.

* cited by examiner

Primary Examiner—David Guzo
Assistant Examiner—Gerald G. Leffers, Jr.
(74) *Attorney, Agent, or Firm*—Knobbe, Martens, Olson & Bear, LLP

(57) **ABSTRACT**

This invention relates to a method for enhancing the production of biologically active proteins and peptides in bacterial cells by infecting bacterial cells of the producer strain, which contain a plasmid with one or more targeted genes, with bacteriophage λ with or without the targeted gene(s). The phage increases synthesis of the targeted protein and induces lysis of the producer strain cells. Super-production is achieved by cultivating the producer strain cells under culture conditions that delay lytic development of the phage. The biologically active proteins and peptides subsequently accumulate in a soluble form in the culture medium as the cells of the producer strain are lysed by the phage.

16 Claims, No Drawings

= WO 200071731 11/30/00

PHAGE-DEPENDENT SUPER-PRODUCTION OF BIOLOGICALLY ACTIVE PROTEIN AND PEPTIDES

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to recombinant DNA technology and more particularly to a new method for enhancing the production of heterologous proteins in bacterial host cells. The disclosed method involves infecting host cells, which contain plasmid encoding the gene of interest, with bacteriophage λ to induce lysis of the bacterial host cells. Superproduction may be achieved in selected host cells either when the plasmid alone carries at least one copy of the heterologous DNA or when both plasmid and phage λ each carry at least one copy of the heterologous DNA.

2. Description of the Related Art

At present, genetic engineering methods allow creating microorganism strains capable of producing substantial amounts of various bioactive substances having important applications in medicine and industry. Typically, plasmid vectors into which a heterologous gene has been inserted are used to transform bacterial host cells. Different strains of *E. coli* are frequently used as recipient cells. Using such plasmid-dependent transformation methods, *E. coli* cells have been engineered to produce a variety of valuable human peptides and proteins, including insulin, γ-interferon, a number of interleukins, superoxidedismutase, plasminogen activator, tumor necrosis factor, erythropoietin, etc. These substances are either already used in medical practice or undergoing different stages of clinical studies.

However, the plasmid method has serious disadvantages. It is technologically complicated, since the desired product has to be extracted from bacterial cells after biosynthesis, which is a multi-stage process. For example, interferon extraction involves disintegration of cells, buffer extraction, polyethylene-imine processing, clarification, precipitation by ammonium sulfate, dialysis, and centrifugation (Goeddel, EP 0043980). The necessity for such extraction and purification steps not only complicates production technology of the recombinant product, but also results in substantial losses, especially during large-scale industrial production.

A further complicating factor is that at relatively high levels of expression of the cloned genes, the eukaryotic proteins generated tend to accumulate in the cytoplasm of *E. coli* as insoluble aggregates, which are often associated with cell membranes. Consequently, the already difficult extraction and purification methods discussed above must be supplemented with additional technical procedures related to the extraction of the insoluble inclusion bodies. Usually, the insoluble proteins are solubilized using ionic detergents, such as SDS or laurylsarcosine, at increased temperatures or in the presence of denaturants, such as 8 M urea or 6–8 M guanidine-HCl.

Often, the final stage of purification involves renaturation and reoxidation of the solubilized polypeptides, which is required to restore functional activity. Disulfide bonds, which are necessary for proper folding of the protein in its native conformation, must be reformed. Renaturation procedures, such as disulfide interchange, may use expensive and relatively toxic reagents, like glutathione, and oxidized 2-mercaptoethanol or dithiothreitol. Further, the final yield of bioactive genetically-engineered proteins may be relatively low. Moreover, the presence of even trace concentrations of the toxic reagents needed to solubilize and then re-establish secondary and tertiary protein structure may prohibit subsequent clinical application of the proteins. Thus, the generation of targeted protein in the form of insoluble inclusion bodies within the bacterial host cells not only complicates the production of recombinant proteins and results in diminished yield, but may also render the final protein unsuitable for clinical use (Fisher, B., Summer, I., Goodenough, P. Biotech. and Bioeng. 41:3–13, 1993).

The technological difficulties associated with the extraction of proteins produced by the expression of heterologous genes from plasmid-transformed bacterial host cells may be overcome by infecting the transformed bacterial host cells with bacteriophage, whose lytic pathway results in lysis of the bearer cell. Thus, the desired protein may be simply released into the culture medium (Breeze A. S. GB 2 143 238A). Accordingly, Breeze disclosed a method of increasing the yield of enzyme produced in *E. coli* by infecting the bacterial cells with phage λ carrying a temperature-sensitive mutation in cI to provide controlled lysis. The cI-gene product is a repressor of early transcription and consequently blocks transcription of the late region of the phage DNA, which is required for head and tail assembly and cell lysis (Mantiatis, T., Fritsch, E. F., Sambrook, J., MOLECULAR CLONING: A LABORATORY MANUAL, 1982, Cold Spring Harbor Laboratory Press). Bacteriophages carrying a temperature-sensitive mutation in cI are able to establish and maintain the lysogenic state as long as the cells are propagated at a temperature that allows the cI-gene product to repress transcription of phage genes necessary for lytic growth. Accordingly, the transformed bacterial host cells may be cultivated at 30° C., wherein the cI-mediated suppression of phage DNA transcription continues and the phage remains in the lysogenic state, until the stage of maximum ferment production is reached. Subsequently, the culture temperature may be increased to 42° C. for 30 minutes in order to inactivate the cI repressor and permit the phage to begin its lytic development. The host cells may then be incubated for 2–3 hours at 30° C. to allow complete lysis and release of the enzyme (Breeze A. S. GB 2 143 238A).

Although Breeze teaches release of proteins from bacterial producer cells, it requires cultivating producers at temperatures not exceeding 30° C., which is not the optimum temperature for growth of *E. coli* cells. Synthesis at the optimum temperature (37° C.) is not possible, since cells at temperatures exceeding 32° C. undergo lysis before reaching the stage of maximum ferment accumulation due to the development of temperature-sensitive lytic prophage. Furthermore, incubation of the bacterial host cells at 42° C. for 30 min as disclosed by Breeze may activate proteases that destroy the targeted protein.

Auerbach et al. (U.S. Pat. No. 4,637,980) used a phage λ DNA fragment for inducing lytic release of recombinant products. In that method, like Breeze, the temperature-sensitive mutation in λ cI-gene product was used to provide temperature-dependent lysis of the bacterial host cells. The λ DNA fragment in Auerbach maintained functional

endolysin-encoding genes, N, Q, R and S, for producing lysozyme following inactivation of the cI repressor at 42° C. Most of the remaining phage genes were deleted; mutations in O and P genes prevented replication of the phage DNA. Consequently, the λ DNA was not a fully functional phage, capable of modulating expression of the targeted gene. Moreover, the λ DNA of Auerbach was not suitable for use as a vector for carrying targeted genes. Further, as discussed above, incubation of the bacterial host cells at 42° to 44° C. for 90–120 min as disclosed by Auerbach may activate proteases that destroy the targeted protein.

In addition to providing for the lytic release of intact protein from bacterial producer cells, bacteriophages have also been used as an alternative to bacterial plasmid-based vectors, for carrying heterologous DNA into host bacterial cells. (Murray, N. E. and Murray, K., *Nature* 251:476–481, 1974; Moir, A., Brammar, W. J., *Molec. gen. Genet.* 149:87–99, 1976). Typically, amplification of genes and their products is achieved during lytic growth of the phage, wherein the phage genome is integrated into the bacterial host DNA (Panasenko, S. M., Cameron, J. R., Davis, R. V., Lehman, L. R., *Science* 196:188–189, 1977; Murray, N. E. and Kelley, W. S., *Molec. gen. Genet.* 175:77–87, 1979; Walter, F., Siegel, M., Malke, H., 1990, DD 276,694; Mory, Y., Revel, M., Chen, L., Sheldon, I. F., Yuti-Chernajovsky, 1983, GB 2,103,222A). Usually, either lysogenic cultures of recombinant phage λ are used for infecting the bacterial host cells, or "warmed up" bacterial cultures, already harboring recombinant lysogenic phage λ, are grown up to amplify expression of the heterologous genes.

Although there are examples of the successful use of λ vectors for expression of heterologous genes, λ vectors have been used primarily for gene cloning. Once cloned, the genes are transferred to plasmid vectors for more effective expression. For example, when *E. coli* is infected by phage λ Charon 4A C15, containing the human β-interferon gene, the quantity of interferon in cell lysate constituted $7–8\times10^6$ units/liter. After the DNA fragment bearing targeted gene was recloned from phage to plasmid, β-interferon yield increased to 1×10^8 units/liter (Moir, A., Brammar, W. J., *Molec. gen. Genet.* 149:87–99, 1976).

To increase the yield of heterologous protein generated in bacterial host cells by recombinant λ vectors, mutations in the phage genome have been introduced that cause phage λ to lose its ability to initiate bacterial cell lysis. Enhanced yield is thereby achieved by extending the period of time during which the heterologous gene is expressed by the bacterial host cells. Thus, for example, the yield of DNA ligase 1 in lysogenic cultures containing λ gt4ligS prophage, with amber-mutation in the S gene, was five times greater than the yield of DNA ligase 1 in lysogenic cultures containing λ gt4lig prophage without the amber-mutation (Panasenko, S. M., Cameron, J. R., Davis, R. V., Lehman, L. R., *Science* 196:188–189, 1977). The phage λ S protein is required for lysis; therefore S⁻mutants accumulate large numbers of intracellular progeny phage particles, as well as the targeted protein, without lysing the host cells (Mantiatis, T., Fritsch, E. F., Sambrook, J., MOLECULAR CLONING: A LABORATORY MANUAL, 1982, Cold Spring Harbor Laboratory Press).

Similar increases in the yield of DNA polymerase 1 were reported for lysogenic cultures containing recombinant phage λ with amber-mutations in the S and Q genes, compared to recombinant phage λ without the amber-mutations (Murray, N. E. and Kelley, W. S., *Molec. gen. Genet.* 175:77–87, 1979). The phage λ Q protein is required for transcription of the late region of the phage DNA, which includes many genes involved in head and tail assembly and cell lysis. (Mantiatis, T., Fritsch, E. F., Sambrook, J., MOLECULAR CLONING: A LABORATORY MANUAL, 1982, Cold Spring Harbor Laboratory Press).

In U.S. Pat. No. 4,710,463, Murray discloses lysogenizing a non-suppressing (Su°) strain of *E. coli* with phage λ containing the temperature-sensitive mutation in cI, as well as mutations in λ S and E genes. Consequently, prolonged cultivation of the lysogenic *E. coli* at 37° C. leads to high levels of production of the recombinant protein, which is retained within the cells, since these are not lysed by phage gene products in the normal way, and since the recombinant phage genome is not encapisdated, it remains available for transcription.

Despite the enhanced yield of heterologous proteins possible using λ-vectors with S and E mutations, the potential technical advantages of bacteriophage vectors related to the lytic release of targeted proteins, may be lost with these mutations, because the targeted proteins accumulate inside the bacterial cell. Thus, when a lysis-defective mutant λ-vector is used for production of heterologous protein, the extraction and purification steps, discussed above for bacterial cells transformed by plasmid vectors, along with the resultant losses, must be performed.

'SUMMARY OF THE INVENTION

The present invention discloses a method for producing a biologically active protein of interest. The method comprises the steps of: (1) transforming a bacterial host cell with a plasmid having at least one copy of an expressible gene encoding the protein, (2) infecting the transformed bacterial host cell with a bacteriophage capable of mediating lysis and also capable of lytic growth without lysis, and (3) cultivating the bacterial host cell under a culture condition that induces lytic growth of the cell without lysis until a desired level of production of the protein is reached.

In a preferred embodiment, the bacteriophage has a temperature-sensitive mutation. More preferably, the bacteriophage is bacteriophage λ and the temperature-sensitive mutation is cI_{857}. The culture condition that induces lytic growth of the bacteriophage is at a temperature of greater than 32° C. Prior to the cultivating step, the bacterial host cells may be grown at a temperature, generally less than about 32° C. that prevents lytic growth of the bacteriophage.

In a variation of the disclosed method, the bacteriophage has a mutation in at least one gene involved in bacteriophage-mediated lysis of the bacterial host cell.

Preferably, the bacteriophage is bacteriophage λ and the at least one gene involved in bacteriophage-mediated lysis is selected from the group consisting of N, Q and R.

Moreover, the bacterial host cell is preferably from a strain of *E. coli*. The strain of *E. coli* may or may not produce a suppressor for the repair of amber-mutations.

Bacteriophage-mediated lysis of the bacterial host cell may be delayed by culturing at higher multiplicities of

infection compared to lower multiplicities of infection. The infecting bacteriophage may be provided at a multiplicity of infection in a range of about 1 to about 100 and more preferably, at a multiplicity of infection in a range of about 10 to about 25.

In another aspect of the present invention, the bacteriophage may contain at least one copy of an expressible gene encoding the same heterologous protein which is encoded by the plasmid.

A variation of the method for producing a biologically active protein in accordance with the present invention is disclosed. The method comprises the steps of: (1) transforming a bacterial host cell with a plasmid having at least one copy of an expressible gene encoding the protein, (2) infecting the transformed bacterial host cell with a bacteriophage having at least one copy of an expressible gene encoding the protein, and (3) cultivating the bacterial host cell under a culture condition that allows expression of the plasmid and phage genes.

In accordance with another aspect of the present invention, a bacterial host cell is disclosed. The bacterial host cell has a plasmid with at least one copy of an expressible heterologous gene encoding a protein, wherein the host cell is infected with a bacteriophage capable of mediating lysis and also capable of lytic growth without lysis.

The bacterial host cell preferably has a bacteriophage with a temperature-sensitive mutation. More preferably, the bacterial host cell is infected with bacteriophage λ and the temperature-sensitive mutation is cI_{857}.

In a variation of the bacterial host cell, the bacteriophage has a mutation in at least one gene involved in bacteriophage-mediated lysis of the host cell. Preferably, the bacterial host cell is infected with bacteriophage λ having a mutation in at least one gene selected from the group consisting of N, Q and R. More preferably, the bacterial host cell is infected with bacteriophage λ with cI_{857}, $Q_{am\ 117}$ and $R_{am\ 54}$ mutations.

In a preferred embodiment of the bacterial host cell of the present invention, the host cell has a plasmid encoding a protein of interest and is also infected with a bacteriophage having at least one copy of an expressible gene encoding the protein of interest.

The bacterial host cell in accordance with the present invention is preferably a strain of *E. coli*. The strain of *E. coli* may or may not have a suppressor for repairing amber-mutations. Similarly, the strain of *E. coli* may or may not be recA deficient. One preferred strain of *E. coli* host cells in accordance with the present invention contains a plasmid having at least one copy of an expressible heterologous gene encoding a protein, wherein the strain of *E. coli* is infected with bacteriophage λ having cI_{857}, $Q_{am\ 117}$ and $R_{am\ 54}$ mutations. The protein may be human alpha-2b interferon. More preferably, in addition to having a plasmid with at least one copy of a gene encoding a protein, the *E. coli* host cell also has a bacteriophage λ having cI_{857}, $Q_{am\ 117}$ and $R_{am\ 54}$ mutations and at least one copy of a gene encoding the protein. This bacteriophage preferably lacks a suppressor for repairing amber-mutations.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Bacteriophage λ is useful as a vector because more than 40% of the viral genome is not essential for lytic growth. This area of the λ genome, located in the central region of the λ DNA, between genes J and N, may be replaced by heterologous DNA encoding a desired product. That region is transcribed early during infection.

In order to maximize the expression of a targeted gene, whose synthesis information is recorded in the area of phage's early genes, special conditions for the phage's development must be provided to ensure proper replication. Further, transcription of the early area, containing the targeted gene, should be fostered, while transcription of the later genes, involved in cell lysis, should be decelerated. This slows down maturation of the λ particles and subsequent cell lysis. Consequently, the early phage products, including the targeted gene product, will accumulate in the bacterial cells. Deceleration of late transcription, thereby extending expression of the targeted gene, may be accomplished by: (1) mutations of phage genome that block expression of the later genes (2) increased multiplicity of infection, and/or (3) cultivation of the infected bacterial cells at a reduced temperature.

An important advantage of infecting producer cells with a bacteriophage is that the phage causes a profound rearrangement of all macromolecular synthesis in the bacterial host cells. By turning off transcription of bacterial genes, phages may increase the copying of the targeted gene, and consequently, increase the output of desired product.

In a preferred embodiment of the present superproduction system, phage λ with amber-mutations that delay bacterial lysis (e.g., Q^- and R^- mutations) are provided in a strain of *E. coli*, designated Su°, which lacks the suppressor responsible for correcting amber-mutations in phage λ. In order to obtain a non-suppressing (Su°) strain of *E. coli*, Su° clones are selected from the wild-type Su^+ population. Preferably, a selection marker is inserted into the phage DNA, e.g., tetracycline or ampicillin resistance.

Selection of Bacterial Strains

Selection of non-suppressing (Su°) strains of *E. coli*, for example, *E. coli* K 802 was carried out with phage λ cI_{857} Nam7Nam53 bla tet (hereinafter λ bla N'). Strain *E. coli* C600 (λ bla N') served as source of the phage. This phage was obtained by insertion of plasmid pCV 11 (bla tet) at EcoRI site into single-site (EcoRI) vector carrying ts-mutation in repressor gene (cI_{857}). Then two amber-mutations were introduced into the phage N gene by recombination in vivo.

Clones were tested for non-lysogenicity with phage λ clear. In addition to phage λ bla N', phage λ cI_{857} Q_{am117} $R_{am\ 54}$ was used to check for suppressor.

Media—Liquid nutrient media, LB and M9 as well as agar medium LB were used for bacterial culture growth (Miller J. H., 1972, Experiments in molecular genetics, Spring Cold Harbor, N.Y.).

Preparation of Phage Lysate—Lysogenic culture was grown in broth at 28° C. under intense aeration to a density of 2×10^8 cells/ml followed by incubation at 43° C. for 20 min. Then it was kept at 37° C. under intense aeration. Cells were lysed in 60–80 min and phage was released into the cultural medium. Phage titer was estimated by a conventional two-layer technique. As a rule, 2×10^{10} PFU/ml of phage lysate were obtained.

As is known, phage λ N' mutant is not able to lyse the host cells and is present in cells in the form of extremely unstable

plasmids. If the host cells contain suppressor, the amber-mutation is phenotypically corrected, the N protein is synthesized and the phage can develop lytically. This difference in the viability of Su^+ and Su° cells, infected by λ N', is used as a basis for selection of spontaneously appearing Su° revertants from the *E. coli* Su^+ cell population. Phage λ with an inserted plasmid that introduced the ampicillin and tetracycline resistance markers into cells was used to prevent the nonlysing Su° cells from masking the search for mutants. The phage also contains ts-mutation in the repressor gene that permits lytic development of such phage resulting in cell lysis.

If the medium supplemented with ampicillin and tetracycline is inoculated with Su^+ culture after its infection with phage λ bla N' with subsequent growth at 43° C., single suppressor-free cells containing phage λ bla N' in the form of plasmids must develop on plates. Su° derivatives of the parent cultures are obtained by curing the cells from the phage. The method can be subdivided into several stages.

1. Infection of Culture With Phage λ bla N'

The culture *E. coli* Su^+ was grown on the M9 medium with maltose at 37° C. under intense agitation to a density of $1–2\times10^8$ cells/ml. The cells were infected with phage λ bla N' at a multiplicity of 5–10 particles per cell and incubated for 20 min at 20° C. Under given conditions, the infection efficiency is about 100%, in addition to the bulk of Su^+ cells, the phage also infects single Su° cells.

2. Selection of Suppressor-Free Cells Containing Marker Phage

After infection, cells were plated out on agar medium supplemented with 12 γ/ml tetracycline and 20 γ/ml ampicillin and grown at 43° C. In 24 h, single colonies developed, which were replated on agar medium with antibiotics and grown at 37° C.

3. Curing of the Selected Clones From Phage λ bla N'

Since phage λ N' in the *E. coli* Su° cells is in the form of extremely unstable plasmids, in order to cure from the phage the selected clones were plated on selective agar medium without antibiotics and grown at 37° C. The number of cells that had lost the phage in the first passage on the medium without antibiotics amounted to 12–35%. The selection of such cells was carried out by monitoring the loss of antibiotic resistance and the acquisition of sensitivity to phage λ clear.

4. Testing of Cells for Repressor

The ability of phage λ with amber-mutations to form plaques on lawns of cured clones was checked. Isogenic suppressor-free derivatives of the parent *E. coli* Su^+ strains are clones, on which phage λ bla N' did not form plaques, phage λ cI_{857} Q_{am117} R_{am54} produced $1–3\times10^{10}$ PFU/ml, and phage λ cI_{857} without mutations in genes Q and R produced 1×10^{10} PFU/ml.

Using this method, Su° revertants of *E. coli* K 802 Su^+ were obtained. Based on the cell number at the moment of infection and the number of Su° revertants among them, the frequency of occurrence of suppressor-free cells was about 3×10^{-7}.

The use of suppressing (Su^+) and non-suppressing (Su°) host strains together with phage λ to achieve superproduction of heterologous proteins and peptides may be more fully understood from the following working examples.

EXAMPLE 1

Increased Synthesis of β-Lactamase in *E. coli* Transformed with pBR322 Carrving the β-Lactamase Gene (bla) following Infection by Phage λ

Bacterial cells, *E. coli* C-600 Su^+, were transformed with plasmid pBR322-bla and cultivated in Aminopeptid medium (manufactured at the Leningrad meat processing and packing factory), diluted 1:1 in 0.15 M NaCl. The C-600 Su^+/pBR322-bla transformants were then grown at 37° C. to a density of 1×10^8 cells/ml and divided into three portions. The control portion was left intact. The second portion was infected with phage λ having the temperature-sensitive mutation in cI, designated λ cI_{857}, and the third portion was infected with phage λ having the temperature-sensitive mutation in cI, as well as amber-mutations in the Q and R genes, designated λ cI_{857} $Q_{am\ 117}$ R_{am54}. Phage mutations were accomplished by standard recombinant method in vivo. Phage multiplicity was approximately 10 phage bodies per 1 bacterial cell. The λ-treated cultures were incubated for 15 min at 37° C. to inactivate the cI repressor, and then for 19 hr at 28° C. The control cultures were incubated at 37° C. for the entire period. β-Lactamase activity was determined by iodometric assay as described by Chaykovakaya, S. M. and Venkina, T. G. Antibiotics 7(5):453–456, 1962. A unit of activity is defined as the minimum quantity of ferment necessary to inactivate 1×10^{-7} M penicillin (60 units) in 1 hr at 37° C., pH 6.8–7.0.

TABLE 1

	Bacterial Cell Culture	β-Lactamase Activity (Units)
1	C-600 Su^+/pBR322-bla	833
2	C-600 Su^+/pBR322-bla + λcI_{857}	4400
3	C-600 Su^+/pBR322-bla + $\lambda cI_{857}Q_{am117}R_{am54}$	8300

As shown in Table 1, β-lactamase synthesis in the C-600 Su^+/pBR322-bla cells infected by phage λ with mutations in the later genes, Q and R, is almost 10 times greater than the synthesis in C-600 Su^+/pBR322-bla control cells.

EXAMPLE 2

Increased Output of β-Lactamase Encoded by bla-Gene Contained in Both Plasmid and Phage

Cultures of *E coli* W 3101 recA⁻13 Su° with and without transformation by pBR322-bla were cultivated in Aminopeptid medium diluted 1:1 with 0.15 M NaCl, at 37° C. to a density of 1×10^8 cells/ml. A recA⁻ strain was used because these cells have a reduced ability to conduct recombination in areas of extended homology in both plasmid and phage (e.g. bla-gene). Thus, recA⁻ cultures avoid exclusion of the homologous bla-gene. The cultures were divided into two portions. The first portion, which was not exposed to phage, was incubated at 37° C. for 16 hr. The second portion was infected with phage λ cI_{587} bla $Q_{am\ 117}$ $R_{am\ 54}$ at a multiplicity of about 10 phage bodies per 1 bacterial cell and cultivated for 2.5–3 hr at 37° C., and then for an additional 14 hr at 28° C. β-Lactamase activity was measured by the iodometric method. The results, shown in Table 2 (below), are expressed in units, as defined above for Table 1.

Phage λ cI_{587} bla $Q_{am\ 117}$ $R_{am\ 54}$ was prepared from lysogenic cultures maintained at 28° C. in Aminopeptid medium. When the bacterial cell density reached about 1×10^8 cells/ml, the cells were warmed for 20 minutes at 43° C. in order to inactivate the cI repressor. Consequently, the prophage is excised from the bacterial genome and begins its lytic development. After 50 min, the cells underwent lysis, releasing 100–200 bodies each. At a density of 1×10^8 cells/ml, the cultures produced $1–2\times10^{10}$ phage bodies per ml. Thus, to infect bacterial cells with phage at a multiplicity of about 10 means that 1 ml of phage lysate (1×10^{10} phage bodies) was added to 10 ml of bacterial suspension (1×10^9 cells).

TABLE 2

	Bacterial Cell Culture	β-Lactamase Activity (Units)
1	W 3101 recA⁻13 Si°/pBR322-bla	13,555
2	W 3101 recA⁻13 Su° + $\lambda cI_{857}blaQ_{am117}R_{am54}$	227,796
3	W 3101 recA⁻13 Su°/pBR322-bla + $\lambda cI_{857}blaQ_{am117}R_{am54}$	2,000,000

As shown in Table 2, bacterial cells which were transformed with both plasmid containing the targeted gene and phage carrying the same gene, produced about 10 times more recombinant protein (β-lactamase) than bacterial cells transformed with phage alone, and over 100 times more β-lactamase than bacterial cells transformed by plasmid alone.

EXAMPLE 3

Super-Production of the β-Galactosidase Encoded by lac-Gene Contained in Both Plasmid and Phage

Cultures of *E. coli* RLM1 containing prophage λ cI_{857} plac5 $Q_{am\ 117}$ $R_{am\ 54}$ (carrying a copy of the β-galactosidase gene, lac5) were grown in LB medium (Difco) at 30° C. with intensive aeration to a density of approximately 1×10^8 cells/ml. The lysogenic culture was warmed to 43° C. and incubated for 20 minutes to inactivate cI repressor. The temperature was then decreased to 37° C. and the bacterial cells underwent lysis, with phages being formed at $1–2\times10^{10}$ PFU/ml. Subsequently, 10 liters of phage lysate, containing about 1×10^{10} phage bodies (λ cI_{857} plac5 $Q_{am\ 117}$ $R_{am\ 54}$) per ml, were added to 40 liters of a suspension of *E coli* Ca 77 Su° transformed by plasmid pZ56 at a density of about 1×10^8 cells/ml in LB medium. Thus, the multiplicity of infection was 25, i.e., there were 25 phage bodies per bacterial cell.

After 7 hr at 37° C., recombinant β-galactosidase constituted 1.9 g per liter of culture medium. The activity of β-galactosidase was calculated by the method of Miller (Miller, J. H., EXPERIMENTS IN MOLECULAR GENETICS, 1972, Cold Spring Harbor Laboratory Press). A unit of activity was calculated as the minimum quantity of ferment required to hydrolyze 1 μM ortho-nitrophenyl-β-D-galactoside to ortho-nitrophenol per min at 30° C., pH 7.0.

EXAMPLE 4

Super-Expression of Human Interferon α-2b

Su⁺ and Su° strains of *E. coli* K 802, transformed with a plasmid bearing a single copy of the gene encoding alpha-2 human interferon (pIF-2-trp), were grown in LB medium to a density of $1.5–2\times10^8$ cells/ml. These cells were then infected at a multiplicity of 15 with different phage λ lysates, as indicated in Table 3 (below). Cultivation continued with intensive aeration at 25° C. for 13 hr. Control cultures, not infected with phage, were incubated at 37° C. for the same period.

TABLE 3

	Bacterial Cell Culture	Phage	Interferon (Units/L)
1	K 802-pIF-2-trp Su⁺		8.0×10^7
2	K 802-pIF-2-trp Su⁺	λcI_{857}	77×10^7
3	K 802-pIF-2-trp Su⁺	$\lambda cI_{857}Q_{am117}R_{am54}$	340×10^7
4	K 802-pIF-2-trp Su⁺	λ-pIF-2-trp $cI_{857}Q_{am117}R_{am54}$	1400×10^7
5	K 802-pIF-2-trp Su°	λ-pIF-2-trp$cI_{857}Q_{am117}R_{am54}$	3000×10^7

As shown in Table 3, when bacterial cells which were transformed with plasmid containing the targeted gene were infected with phage λ containing only the temperature-sensitive mutation in cI, interferon expression increased by about 10-fold compared to control, non-infected cultures. Adding the amber-mutations in Q and R genes further increased expression by about 40-fold compared to control bacterial cells.

Adding a copy of the interferon gene to phage λ with cI, Q and R mutations increased interferon synthesis by 175-fold over controls. Finally, when a non-suppressing, Su°, strain of *E. coli*, transformed by a plasmid bearing a copy of the interferon gene, was infected with phage λ, also having a copy of the interferon gene, as well as cI, Q and R mutations, the bacterial host cells produced about 375 times more recombinant protein than the control cells transformed by plasmid alone.

EXAMPLE 5

Enhanced Recovery of Biologically Active Recombinant Interferon by Phage-Mediated Host Cell Lysis

Strain *E. Coli* SG 20050 was transformed by a plasmid bearing two copies of the human interferon alpha-2b gene (pIF-14) by standard methods. The transformant cells were grown up in 80 liters of LB medium at 37° C. with intensive aeration to a density of 2×10^8 cells/ml. The culture was divided into two portions. The first was not infected with phage. The second was infected with phage λ lysate harvested from *E. coli* K 802/λ cI_{857} $Q_{am\ 117}$ $R_{am\ 54}$ at a multiplicity of 10 phage bodies per bacterial cell. The control cells were incubated for 19 hr at 37° C. and the phage-infected cells were incubated for 19 hr at 21° C.

Interferon production in both control and phage-infected cultures was about 20% of the total cellular protein. However, the interferon in control cells was associated at least in part with insoluble inclusion bodies. Thus, it was not possible to determine its biological activity without solubilization, denaturation and renaturation. In contrast, the specific activity of the soluble interferon released into the medium following phage-mediated cell lysis, was readily determined by standard immunoenzyme assay. The interferon activity was 4×10^{10} IU/liter (200 mg/liter).

Pre-clinical toxicological studies of recombinant human alpha-2β interferon produced by the phage super-production method of the present invention showed that the compound was practically non-toxic. Intra-abdominal and intramuscular injections of the recombinant interferon in white mice and Wistar rats at 8.5×10^9 ME/kg (2.5×10^5 times the maximum human dose) and intravenous injections in mice and rabbits at 4.25×10^9 ME/kg (1.25×10^5 times the human therapeutic dose) produced no pronounced intoxication or death of the animals. Four months of injections in Wistar rats at 6×10^5, 6×10^6 and 3×10^7 ME/kg (18, 180 and 900 times the human therapeutic dose, respectively) showed no damage to the main organs and bodily systems of the experimental animals. Likewise, three months of intravenous injections in rabbits of 6×10^5 and 6×10^6 ME/kg, and two months of intramuscular injections in dogs at 3×10^6 ME/kg showed no signs of damage to the organ systems.

During immunotoxic and allergenic analysis of recombinant interferon, the induction of cellular and humoral immunity reactions, as well as delayed and immediate hypersensitivity reactions were studied. The results indicated that no immunotoxic or allergenic influence was produced. The recombinant interferon also had no mutagenic or DNA-damaging effects in bacteria during metabolic activation in vitro or in bone marrow of mouse embryos in vivo.

Embryotoxic studies of recombinant interferon were conducted in pregnant hamadryad baboon females. Daily intramuscular doses during organogenesis (20^{th} to 50^{th} days of pregnancy) caused defects in embryo development leading to miscarriage or stillbirth. Similar results were obtained for recombinant interferon analogs, and most probably could be explained by a powerful antiproliferative action of interferons. It is possible that the miscarriage may be attributed to a "cancellation" of immunologic tolerance of maternal organism towards the fetus, caused by immuno-modulating action of the protein.

Recombinant interferon was also studied in Ukrainian clinics. Based on these clinical studies, the recombinant interferon was shown to be useful in the treatment of a variety of human diseases and conditions. For example, recombinant interferon was effective in treating acute and chronic hepatitis B, acute viral, bacterial and mixed infections, acute and chronic septic diseases, herpetic infections, herpes zoster, papillomatosis of larynx, multiple sclerosis, and various cancers, including melanoma, renal cell carcinoma, bladder carcinoma, ovarian carcinoma, breast cancer, Kaposi's sarcoma and myeloma.

The contraindications in human clinical applications were prolonged (several months) use at high doses, allergy and pregnancy. The possible side effects noted were small and transitory "flu-like" symptoms and at prolonged regimens, leuko and trombocytopenia were marked.

While we have described a number of embodiments of this invention, it is A apparent that our description of the invention can be altered to provide other embodiments that utilize the basic process of this invention. Therefore, it will be appreciated by those of skill in the art that the scope of this invention is to be defined by the claims appended hereto rather than the specific embodiments that have been described in detail above by way of example.

What is claimed is:

1. An *E. coli* host cell with a plasmid having at least one copy of an expressible eukaryotic gone encoding a protein, wherein said *E. coli* host cell is lytically infected with bacteriophage λ having cI_{857}, Q_{am117}, and R_{am54} mutations.

2. The host cell of claim **1**, wherein said protein is human alpha-2b interferon.

3. The host cell of claim **1**, wherein said *E. coli* host cell lacks a suppressor for repairing amber-mutations.

4. The host cell of claim **1**, further comprising recA⁻13.

5. An *E. coli* host cell with a plasmid having at least one copy of an expressible eukaryotic gene encoding a protein, wherein said *E. coli* host cell is lytically infected with bacteriophage λ having cI_{857}, Q_{am117}, and R_{am54} mutations and at least one copy of an expressible eukaryotic gene encoding said protein.

6. The host cell of claim **5**, wherein said *E. coli* host cell lacks a suppressor for repairing amber-mutations.

7. The host cell of claim **4**, wherein said protein is human alpha-2b interferon.

8. An *E. coli* host cell with a plasmid having at least one copy of an expressible eukaryotic gene encoding a protein, wherein said *E. coli* host cell is lytically infected with a bacteriophage λ, wherein the bacteriophage λ has at least one mutated gene selected from the group consisting of N, Q and R.

9. The *E. coli* host cell of claim **8**, wherein the bacteriophage λ has a temperature-sensitive mutation.

10. The *E. coli* host cell of claim **9**, wherein the temperature-sensitive mutation is cI_{857}.

11. The *E. coli* host cell of claim **8** which lacks a suppressor for repairing amber-mutations.

12. The *E. coli* host cell of claim **8** which is recA deficient.

13. The host cell of claim **8**, wherein said protein is human alpha-2b interferon.

14. An *E. coli* host cell with a plastmid having at least one copy of an expressible heterologous en totic gene encoding a protein, wherein said *E. coli* host cell is lytically infected with bactetophage lambda, wherein the bacteriophage lambda has at least one mutated gene selected from the group consisting of N, Q, and R and at least one copy of an expressible heterologous eukaryotic gene encoding said protein.

15. The host cell of claim **14**, wherein said protein is human alpha-2b interferon.

16. The host cell of claim **14**, wherein said *E. coli* host cell lacks a suppressor for repairing amber-mutations.

* * * * *

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 February 2002 (21.02.2002)

PCT

(10) International Publication Number
WO 02/14471 A2

(51) International Patent Classification[7]: C12N

(21) International Application Number: PCT/US01/25537

(22) International Filing Date: 15 August 2001 (15.08.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/225,406 15 August 2000 (15.08.2000) US

(71) Applicant: **PHAGE BIOTECHNOLOGY CORPORATION** [US/US]; 14272 Franklin Avenue, Suite 100, Tustin, CA 92780 (US).

(72) Inventors: **STEGMANN, Thomas, J.**; Spiegel Strasse #10, 36100 Petersberg (DE). **KORDYUM, Vitaliy, A.**; Artyoma Street 53, Kiev-053, 254053 Ukraine (UA). **CHERNYKH, Svitlana, I.**; Lomonosova Street 29, Apt. 6, Kiev-127, 252127 Ukraine (UA). **SLAVCHENKO, Iryna, Yu.**; Revutskogo Street 44, Apt. 81, Kiev-068, 253068 Ukraine (UA). **VOZIANOV, Oleksandr, F.**; Desyatinnaya Street 10, Apt. 6, Kiev-025, 252025 Ukraine (UA).

PCT - Hausel

(74) Agent: **ALTMAN, Daniel, E.**; KNOBBE, MARTENS, OLSON & BEAR, LLP, 620 Newport Center Drive, Sixteenth Floor, Newport Beach, CA 92660 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EC, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: A METHOD OF PRODUCING BIOLOGICALLY ACTIVE HUMAN ACIDIC FIBROBLAST GROWTH FACTOR AND ITS USE IN PROMOTING ANGIOGENESIS

(57) Abstract: The gene of human acidic fibroblast growth factor 155 (haFGF 155) has been obtained by chemical synthesis. The nucleotide sequence of haFGF 155 gene has been deduced on the basis of haFGF 155 amino acid sequence as described in the literature. The amino acid sequence of the synthesized haFGF 155 does not differ from those described in the literature. The nucleotide sequence of haFGF gene differs from those described previously. For chemical synthesis of haFGF 155 gene, codons were used which are the ones most often used by E. coli in highly expressed E. coli proteins. A plasmid with haFGF 155 (phaFGF 155) gene was obtained and was used to transform E. coli. Production of haFGF 154 protein was achieved by cultivation of the producer strain under conditions which slow down the lytic development of lambda phage. The haFGF 154 protein accumulated in culture medium in a soluble condition as a result of the producer strain cells lysis by the lambda phage. The haFGF 154 protein constituted 20% of the soluble protein accumulated in the culture medium and its biological activity was demonstrated by its ability to generate new vessels (angiogenesis). The initiator methionine residue at position 1 of the FGF 155 protein was completely removed during protein synthesis resulting in an FGF 154 amino acid product. The use of the phage-dependent method to produce other forms of the haFGF protein is also disclosed.

WO 02/14471 A2

11/7/22
DIALOG(R)File 155:MEDLINE(R)

04833876 85140231 PMID: 3156376
A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes.
Tabor S; Richardson C C
Proceedings of the National Academy of Sciences of the United States of America (UNITED STATES) Feb 1985, 82 (4) p1074-8, ISSN 0027-8424
Journal Code: 7505876
Contract/Grant No.: AI-06045; AI; NIAID
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The RNA polymerase gene of bacteriophage T7 has been cloned into the plasmid pBR322 under the inducible control of the lambda PL promoter. After induction, T7 RNA polymerase constitutes 20% of the soluble protein of Escherichia coli, a 200-fold increase over levels found in T7-infected cells. The overproduced enzyme has been purified to homogeneity. During extraction the enzyme is sensitive to a specific proteolysis, a reaction that can be prevented by a modification of lysis conditions. The specificity of T7 RNA polymerase for its own promoters, combined with the ability to inhibit selectively the host RNA polymerase with rifampicin, permits the exclusive expression of genes under the control of a T7 RNA polymerase promoter. We describe such a coupled system and its use to express high levels of phage T7 gene 5 protein, a subunit of T7 DNA polymerase.
Record Date Created: 19850405
Record Date Completed: 19850405

Restricted, other claims. No DP.

US 20010044133A1

(19) United States

(12) Patent Application Publication (10) Pub. No.: US 2001/0044133 A1

Kordyum et al. (43) Pub. Date: Nov. 22, 2001

(54) PHAGE-DEPENDENT SUPER-PRODUCTION OF BIOLOGICALLY ACTIVE PROTEIN AND PEPTIDES

(76) Inventors: **Vitaliy A. Kordyum**, Kiev (UA); **Svitlana I. Chernykh**, Kiev (UA); **Iryna Yu Slavchenko**, Kiev (UA); **Oleksandr F. Vozlanov**, Kiev (UA)

Correspondence Address:
KNOBBE MARTENS OLSON & BEAR LLP
620 NEWPORT CENTER DRIVE
SIXTEENTH FLOOR
NEWPORT BEACH, CA 92660 (US)

(21) Appl. No.: **09/859,651**

(22) Filed: **May 17, 2001**

Related U.S. Application Data

(62) Division of application No. 09/318,288, filed on May 25, 1999, now Pat. No. 6,268,178.

Publication Classification

(51) **Int. Cl.**[7] **C12P 21/02**; C12N 7/01; C12N 1/20; C12N 15/74

(52) **U.S. Cl.** **435/69.1**; 435/252.33; 435/477; 435/235.1

(57) **ABSTRACT**

This invention relates to a method for enhancing the production of biologically active proteins and peptides in bacterial cells by infecting bacterial cells of the producer strain, which contain a plasmid with one or more targeted genes, with bacteriophage λ with or without the targeted gene(s). The phage increases synthesis of the targeted protein and induces lysis of the producer strain cells. Super-production is achieved by cultivating the producer strain cells under culture conditions that delay lytic development of the phage. The biologically active proteins and peptides subsequently accumulate in a soluble form in the culture medium as the cells of the producer strain are lysed by the phage.

PHAGE-DEPENDENT SUPER-PRODUCTION OF BIOLOGICALLY ACTIVE PROTEIN AND PEPTIDES

BACKGROUND OF THE INVENTION

[0001] 1. Field of the Invention

[0002] This invention relates to recombinant DNA technology and more particularly to a new method for enhancing the production of heterologous proteins in bacterial host cells. The disclosed method involves infecting host cells, which contain plasmid encoding the gene of interest, with bacteriophage λ to induce lysis of the bacterial host cells. Super-production may be achieved in selected host cells either when the plasmid alone carries at least one copy of the heterologous DNA or when both plasmid and phage λ each carry at least one copy of the heterologous DNA.

[0003] 2. Description of the Related Art

[0004] At present, genetic engineering methods allow creating microorganism strains capable of producing substantial amounts of various bioactive substances having important applications in medicine and industry. Typically, plasmid vectors into which a heterologous gene has been inserted are used to transform bacterial host cells. Different strains of *E. coli* are frequently used as recipient cells. Using such plasmid-dependent transformation methods, *E. coli* cells have been engineered to produce a variety of valuable human peptides and proteins, including insulin, γ-interferon, a number of interleukins, superoxidedismutase, plasminogen activator, tumor necrosis factor, erythropoietin, etc. These substances are either already used in medical practice or undergoing different stages of clinical studies.

[0005] However, the plasmid method has serious disadvantages. It is technologically complicated, since the desired product has to be extracted from bacterial cells after biosynthesis, which is a multi-stage process. For example, interferon extraction involves disintegration of cells, buffer extraction, polyethylene-imine processing, clarification, precipitation by ammonium sulfate, dialysis, and centrifugation (Goeddel, EP 0043980). The necessity for such extraction and purification steps not only complicates production technology of the recombinant product, but also results in substantial losses, especially during large-scale industrial production.

[0006] A further complicating factor is that at relatively high levels of expression of the cloned genes, the eukaryotic proteins generated tend to accumulate in the cytoplasm of *E. coli* as insoluble aggregates, which are often associated with cell membranes. Consequently, the already difficult extraction and purification methods discussed above must be supplemented with additional technical procedures related to the extraction of the insoluble inclusion bodies. Usually, the insoluble proteins are solubilized using ionic detergents, such as SDS or laurylsarcosine, at increased temperatures or in the presence of denaturants, such as 8 M urea or 6-8 M guanidine-HCl.

[0007] Often, the final stage of purification involves renaturation and reoxidation of the solubilized polypeptides, which is required to restore functional activity. Disulfide bonds, which are necessary for proper folding of the protein in its native conformation, must be reformed. Renaturation procedures, such as disulfide interchange, may use expensive and relatively toxic reagents, like glutathione, and oxidized 2-mercaptoethanol or dithiothreitol. Further, the final yield of bioactive genetically-engineered proteins may be relatively low. Moreover, the presence of even trace concentrations of the toxic reagents needed to solubilize and then re-establish secondary and tertiary protein structure may prohibit subsequent clinical application of the proteins. Thus, the generation of targeted protein in the form of insoluble inclusion bodies within the bacterial host cells not only complicates the production of recombinant proteins and results in diminished yield, but may also render the final protein unsuitable for clinical use (Fisher, B., Sumner, I., Goodenough, P. Biotech. and Bioeng. 41:3-13, 1993).

[0008] The technological difficulties associated with the extraction of proteins produced by the expression of heterologous genes from plasmid-transformed bacterial host cells may be overcome by infecting the transformed bacterial host cells with bacteriophage, whose lytic pathway results in lysis of the bearer cell. Thus, the desired protein may be simply released into the culture medium (Breeze A. S. GB 2 143 238A). Accordingly, Breeze disclosed a method of increasing the yield of enzyme produced in *E. coli* by infecting the bacterial cells with phage λ carrying a temperature-sensitive mutation in cI to provide controlled lysis. The cI-gene product is a repressor of early transcription and consequently blocks transcription of the late region of the phage DNA, which is required for head and tail assembly and cell lysis (Mantiatis, T., Fritsch, E. F., Sambrook, J., MOLECULAR CLONING: A LABORATORY MANUAL, 1982, Cold Spring Harbor Laboratory Press). Bacteriophages carrying a temperature-sensitive mutation in cI are able to establish and maintain the lysogenic state as long as the cells are propagated at a temperature that allows the cI-gene product to repress transcription of phage genes necessary for lytic growth. Accordingly, the transformed bacterial host cells may be cultivated at 30° C., wherein the cI-mediated suppression of phage DNA transcription continues and the phage remains in the lysogenic state, until the stage of maximum ferment production is reached. Subsequently, the culture temperature may be increased to 42° C. for 30 minutes in order to inactivate the cI repressor and permit the phage to begin its lytic development. The host cells may then be incubated for 2-3 hours at 30° C. to allow complete lysis and release of the enzyme (Breeze A. S. GB 2 143 238A).

[0009] Although Breeze teaches release of proteins from bacterial producer cells, it requires cultivating producers at temperatures not exceeding 30° C., which is not the optimum temperature for growth of *E. coli* cells. Synthesis at the optimum temperature (37° C.) is not possible, since cells at temperatures exceeding 32° C. undergo lysis before reaching the stage of maximum ferment accumulation due to the development of temperature-sensitive lytic prophage. Furthermore, incubation of the bacterial host cells at 42° C. for 30 min as disclosed by Breeze may activate proteases that destroy the targeted protein.

[0010] Auerbach et al. (U.S. Pat. No. 4,637,980) used a phage λ DNA fragment for inducing lytic release of recombinant products. In that method, like Breeze, the temperature-sensitive mutation in λ cI-gene product was used to provide temperature-dependent lysis of the bacterial host cells. The λ DNA fragment in Auerbach maintained functional endolysin-encoding genes, N, Q, R and S, for producing lysozyme following inactivation of the cI repressor at

42° C. Most of the remaining phage genes were deleted; mutations in O and P genes prevented replication of the phage DNA. Consequently, the λ DNA was not a fully functional phage, capable of modulating expression of the targeted gene. Moreover, the λ DNA of Auerbach was not suitable for use as a vector for carrying targeted genes. Further, as discussed above, incubation of the bacterial host cells at 42° to 44° C. for 90-120 min as disclosed by Auerbach may activate proteases that destroy the targeted protein.

[0011] In addition to providing for the lytic release of intact protein from bacterial producer cells, bacteriophages have also been used as an alternative to bacterial plasmid-based vectors, for carrying heterologous DNA into host bacterial cells. (Murray, N. E. and Murray, K., *Nature* 251:476-481, 1974; Moir, A., Brammar, W. J., *Molec. gen. Genet.* 149:87-99, 1976). Typically, amplification of genes and their products is achieved during lytic growth of the phage, wherein the phage genome is integrated into the bacterial host DNA (Panasenko, S. M., Cameron, J. R., Davis, R. V., Lehman, L. R., *Science* 196:188-189, 1977; Murray, N. E. and Kelley, W. S., *Molec. gen. Genet.* 175:77-87, 1979; Walter, F., Siegel, M., Malke, H., 1990, DD 276,694; Mory, Y., Revel, M., Chen, L., Sheldon, I. F., Yuti-Chernajovsky, 1983, GB 2,103,222A). Usually, either lysogenic cultures of recombinant phage λ are used for infecting the bacterial host cells, or "warmed up" bacterial cultures, already harboring recombinant lysogenic phage λ., are grown up to amplify expression of the heterologous genes.

[0012] Although there are examples of the successful use of λ vectors for expression of heterologous genes, λ vectors have been used primarily for gene cloning. Once cloned, the genes are transferred to plasmid vectors for more effective expression. For example, when *E. coli* is infected by phage λ Charon 4A C15, containing the human β-interferon gene, the quantity of interferon in cell lysate constituted $7\text{-}8\times10^6$ units/liter. After the DNA fragment bearing targeted gene was recloned from phage to plasmid, β-interferon yield increased to 1×10^8 units/liter (Moir, A., Brammar, W. J., *Molec. gen. Genet.* 149:87-99, 1976).

[0013] To increase the yield of heterologous protein generated in bacterial host cells by recombinant λ vectors, mutations in the phage genome have been introduced that cause phage λ to lose its ability to initiate bacterial cell lysis. Enhanced yield is thereby achieved by extending the period of time during which the heterologous gene is expressed by the bacterial host cells. Thus, for example, the yield of DNA ligase 1 in lysogenic cultures containing λ gt4ligS prophage, with amber-mutation in the S gene, was five times greater than the yield of DNA ligase 1 in lysogenic cultures containing λ gt4lig prophage without the amber-mutation (Panasenko, S. M., Cameron, J. R., Davis, R. V., Lehman, L. R., *Science* 196:188-189, 1977). The phage λ S protein is required for lysis; therefore S^- mutants accumulate large numbers of intracellular progeny phage particles, as well as the targeted protein, without lysing the host cells (Mantiatis, T., Fritsch, E. F., Sambrook, J., MOLECULAR CLONING: A LABORATORY MANUAL, 1982, Cold Spring Harbor Laboratory Press).

[0014] Similar increases in the yield of DNA polymerase 1 were reported for lysogenic cultures containing recombinant phage λ with amber-mutations in the S and Q genes, compared to recombinant phage λ without the amber-mutations (Murray, N. E. and Kelley, W. S., *Molec. gen. Genet.* 175:77-87, 1979). The phage λ Q protein is required for transcription of the late region of the phage DNA, which includes many genes involved in head and tail assembly and cell lysis. (Mantiatis, T., Fritsch, E. F., Sambrook, J., MOLECULAR CLONING: A LABORATORY MANUAL, 1982, Cold Spring Harbor Laboratory Press).

[0015] In U.S. Pat. No. 4,710,463, Murray discloses lysogenizing a non-suppressing (Su°) strain of *E. coli* with phage λ containing the temperature-sensitive mutation in cI, as well as mutations in λ S and E genes. Consequently, prolonged cultivation of the lysogenic *E. coli* at 37° C. leads to high levels of production of the recombinant protein, which is retained within the cells, since these are not lysed by phage gene products in the normal way, and since the recombinant phage genome is not encapisdated, it remains available for transcription.

[0016] Despite the enhanced yield of heterologous proteins possible using λ-vectors with S and E mutations, the potential technical advantages of bacteriophage vectors related to the lytic release of targeted proteins, may be lost with these mutations, because the targeted proteins accumulate inside the bacterial cell. Thus, when a lysis-defective mutant λ-vector is used for production of heterologous protein, the extraction and purification steps, discussed above for bacterial cells transformed by plasmid vectors, along with the resultant losses, must be performed.

SUMMARY OF THE INVENTION

[0017] The present invention discloses a method for producing a biologically active protein of interest. The method comprises the steps of: (1) transforming a bacterial host cell with a plasmid having at least one copy of an expressible gene encoding the protein, (2) infecting the transformed bacterial host cell with a bacteriophage capable of mediating lysis and also capable of lytic growth without lysis, and (3) cultivating the bacterial host cell under a culture condition that induces lytic growth of the cell without lysis until a desired level of production of the protein is reached.

[0018] In a preferred embodiment, the bacteriophage has a temperature-sensitive mutation. More preferably, the bacteriophage is bacteriophage λ and the temperature-sensitive mutation is cI_{857}. The culture condition that induces lytic growth of the bacteriophage is at a temperature of greater than 32° C. Prior to the cultivating step, the bacterial host cells may be grown at a temperature, generally less than about 32° C. that prevents lytic growth of the bacteriophage.

[0019] In a variation of the disclosed method, the bacteriophage has a mutation in at least one gene involved in bacteriophage-mediated lysis of the bacterial host cell. Preferably, the bacteriophage is bacteriophage λ and the at least one gene involved in bacteriophage-mediated lysis is selected from the group consisting of N, Q and R. Moreover, the bacterial host cell is preferably from a strain of *E. coli*. The strain of *E. coli* may or may not produce a suppressor for the repair of amber-mutations.

[0020] Bacteriophage-mediated lysis of the bacterial host cell may be delayed by culturing at higher multiplicities of infection compared to lower multiplicities of infection. The

infecting bacteriophage may be provided at a multiplicity of infection in a range of about 1 to about 100 and more preferably, at a multiplicity of infection in a range of about 10 to about 25.

[0021] In another aspect of the present invention, the bacteriophage may contain at least one copy of an expressible gene encoding the same heterologous protein which is encoded by the plasmid.

[0022] A variation of the method for producing a biologically active protein in accordance with the present invention is disclosed. The method comprises the steps of: (1) transforming a bacterial host cell with a plasmid having at least one copy of an expressible gene encoding the protein, (2) infecting the transformed bacterial host cell with a bacteriophage having at least one copy of an expressible gene encoding the protein, and (3) cultivating the bacterial host cell under a culture condition that allows expression of the plasmid and phage genes.

[0023] In accordance with another aspect of the present invention, a bacterial host cell is disclosed. The bacterial host cell has a plasmid with at least one copy of an expressible heterologous gene encoding a protein, wherein the host cell is infected with a bacteriophage capable of mediating lysis and also capable of lytic growth without lysis.

[0024] The bacterial host cell preferably has a bacteriophage with a temperature-sensitive mutation. More preferably, the bacterial host cell is infected with bacteriophage λ and the temperature-sensitive mutation is cI_{857}.

[0025] In a variation of the bacterial host cell, the bacteriophage has a mutation in at least one gene involved in bacteriophage-mediated lysis of the host cell. Preferably, the bacterial host cell is infected with bacteriophage λ having a mutation in at least one gene selected from the group consisting of N, Q and R. More preferably, the bacterial host cell is infected with bacteriophage λ with cI_{857}, $Q_{am\ 117}$ and $R_{am\ 54}$ mutations.

[0026] In a preferred embodiment of the bacterial host cell of the present invention, the host cell has a plasmid encoding a protein of interest and is also infected with a bacteriophage having at least one copy of an expressible gene encoding the protein of interest.

[0027] The bacterial host cell in accordance with the present invention is preferably a strain of *E. coli*. The strain of *E. coli* may or may not have a suppressor for repairing amber-mutations. Similarly, the strain of *E. coli* may or may not be recA deficient. One preferred strain of *E. coli* host cells in accordance with the present invention contains a plasmid having at least one copy of an expressible heterologous gene encoding a protein, wherein the strain of *E. coli* is infected with bacteriophage λ having cI_{857}, $Q_{am\ 117}$ and $R_{am\ 54}$ mutations. The protein may be human alpha-2b interferon. More preferably, in addition to having a plasmid with at least one copy of a gene encoding a protein, the *E. coli* host cell also has a bacteriophage λ having cI_{857}, $Q_{am\ 117}$ and $R_{am\ 54}$ mutations and at least one copy of a gene encoding the protein. This bacteriophage preferably lacks a suppressor for repairing amber-mutations.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0028] Bacteriophage λ is useful as a vector because more than 40% of the viral genome is not essential for lytic growth. This area of the λ genome, located in the central region of the λ DNA, between genes J and N, may be replaced by heterologous DNA encoding a desired product. That region is transcribed early during infection.

[0029] In order to maximize the expression of a targeted gene, whose synthesis information is recorded in the area of phage's early genes, special conditions for the phage's development must be provided to ensure proper replication. Further, transcription of the early area, containing the targeted gene, should be fostered, while transcription of the later genes, involved in cell lysis, should be decelerated. This slows down maturation of the λ particles and subsequent cell lysis. Consequently, the early phage products, including the targeted gene product, will accumulate in the bacterial cells. Deceleration of late transcription, thereby extending expression of the targeted gene, may be accomplished by: (1) mutations of phage genome that block expression of the later genes (2) increased multiplicity of infection, and/or (3) cultivation of the infected bacterial cells at a reduced temperature.

[0030] An important advantage of infecting producer cells with a bacteriophage is that the phage causes a profound rearrangement of all macromolecular synthesis in the bacterial host cells. By turning off transcription of bacterial genes, phages may increase the copying of the targeted gene, and consequently, increase the output of desired product.

[0031] In a preferred embodiment of the present superproduction system, phage λ with amber-mutations that delay bacterial lysis (e.g., Q^- and R^- mutations) are provided in a strain of *E. coli*, designated Su°, which lacks the suppressor responsible for correcting amber-mutations in phage λ. In order to obtain a non-suppressing (Su°) strain of *E. coli*, Su° clones are selected from the wild-type Su^+ population. Preferably, a selection marker is inserted into the phage DNA, e.g., tetracycline or ampicillin resistance.

[0032] Selection of Bacterial Strains

[0033] Selection of non-suppressing (Su°) strains of *E. coli*, for example, *E. coli* K 802 was carried out with phage λ cI_{857} Nam7Nam53 bla tet (hereinafter λ bla N'). Strain *E. coli* C600 (λ bla N') served as source of the phage. This phage was obtained by insertion of plasmid pCV 11 (bla tet) at EcoRI site into single-site (EcoRI) vector carrying ts-mutation in repressor gene (cI_{857}) Then two amber-mutations were introduced into the phage N gene by recombination in vivo.

[0034] Clones were tested for non-lysogenicity with phage λ clear. In addition to phage λ bla N', phage λ cI_{857} $Q_{am\ 117}R_{am\ 54}$ was used to check for suppressor.

[0035] Media—Liquid nutrient media, LB and M9 as well as agar medium LB were used for bacterial culture growth (Miller J. H., 1972, Experiments in molecular genetics, Spring Cold Harbor, N.Y.).

[0036] Preparation of Phage Lysate—Lysogenic culture was grown in broth at 28° C. under intense aeration to a density of 2×10^8 cells/ml followed by incubation at 43° C. for 20 min. Then it was kept at 37° C. under intense aeration. Cells were lysed in 60-80 min and phage was released into the cultural medium. Phage titer was estimated by a conventional two-layer technique. As a rule, 2×10^{10} PFU/ml of phage lysate were obtained.

[0037] As is known, phage λ N' mutant is not able to lyse the host cells and is present in cells in the form of extremely unstable plasmids. If the host cells contain suppressor, the amber-mutation is phenotypically corrected, the N protein is synthesized and the phage can develop lytically. This difference in the viability of Su^+ and Su^- cells, infected by λ N', is used as a basis for selection of spontaneously appearing Su^- revertants from the *E. coli* Su^+ cell population. Phage λ with an inserted plasmid that introduced the ampicillin and tetracycline resistance markers into cells was used to prevent the nonlysing Su^- cells from masking the search for mutants. The phage also contains ts-mutation in the repressor gene that permits lytic development of such phage resulting in cell lysis.

[0038] If the medium supplemented with ampicillin and tetracycline is inoculated with Su^+ culture after its infection with phage λ bla N' with subsequent growth at 43° C., single suppressor-free cells containing phage λ bla N' in the form of plasmids must develop on plates. Su^- derivatives of the parent cultures are obtained by curing the cells from the phage. The method can be subdivided into several stages.

[0039] 1. Infection of Culture With Phage λ bla N'

[0040] The culture *E. coli* Su^+ was grown on the M9 medium with maltose at 37° C. under intense agitation to a density of $1\text{-}2\times10^8$ cells/ml. The cells were infected with phage λ bla N' at a multiplicity of 5-10 particles per cell and incubated for 20 min at 20° C. Under given conditions, the infection efficiency is about 100%, in addition to the bulk of Su^+ cells, the phage also infects single Su^- cells.

[0041] 2. Selection of Suppressor-Free Cells Containing Marker Phage

[0042] After infection, cells were plated out on agar medium supplemented with 12 γ/ml tetracycline and 20 γ/ml ampicillin and grown at 43° C. In 24 h, single colonies developed, which were replated on agar medium with antibiotics and grown at 37° C.

[0043] 3. Curing of the Selected Clones From Phage λ bla N'

[0044] Since phage λ N' in the *E. coli* Su^- cells is in the form of extremely unstable plasmids, in order to cure from the phage the selected clones were plated on selective agar medium without antibiotics and grown at 37° C. The number of cells that had lost the phage in the first passage on the medium without antibiotics amounted to 12-35%. The selection of such cells was carried out by monitoring the loss of antibiotic resistance and the acquisition of sensitivity to phage λ clear.

[0045] 4. Testing of Cells for Repressor

[0046] The ability of phage λ with amber-mutations to form plaques on lawns of cured clones was checked. Isogenic suppressor-free derivatives of the parent *E. coli* Su^+ strains are clones, on which phage λ bla N' did not form plaques, phage λ cI_{857} $Q_{am\ 117}$ $R_{am\ 54}$ produced $1\text{-}3\times10^5$ PFU/ml, and phage λ cI_{857} without mutations in genes Q and R produced 1×10^{10} PFU/ml.

[0047] Using this method, Su^- revertants of *E. coli* K 802 Su^+ were obtained. Based on the cell number at the moment of infection and the number of Su^- revertants among them, the frequency of occurrence of suppressor-free cells was about 3×10^{-7}.

[0048] The use of suppressing (Su^+) and non-suppressing (Su^-) host strains together with phage λ to achieve superproduction of heterologous proteins and peptides may be more fully understood from the following working examples.

EXAMPLE 1

Increased Synthesis of β-Lactamase in *E. coli* Transformed with pBR322 Carrying the β-Lactamase Gene (bla) following Infection by Phage λ

[0049] Bacterial cells, *E. coli* C-600 Su^+, were transformed with plasmid pBR322-bla and cultivated in Aminopeptid medium (manufactured at the Leningrad meat processing and packing factory), diluted 1:1 in 0.15 M NaCl. The C-600 Su^+/pBR322-bla transformants were then grown at 37° C. to a density of 1×10^8 cells/ml and divided into three portions. The control portion was left intact. The second portion was infected with phage λ having the temperature-sensitive mutation in cI, designated λ cI_{857}, and the third portion was infected with phage λ having the temperature-sensitive mutation in cI, as well as amber-mutations in the Q and R genes, designated λ cI_{857} $Q_{am\ 117}R_{am\ 54}$. Phage mutations were accomplished by standard recombinant method in vivo. Phage multiplicity was approximately 10 phage bodies per 1 bacterial cell. The λ-treated cultures were incubated for 15 min at 37° C. to inactivate the cI repressor, and then for 19 hr at 28° C. The control cultures were incubated at 37° C. for the entire period. β-Lactamase activity was determined by iodometric assay as described by Chaykovakaya, S. M. and Venkina, T. G. Antibiotics 7(5):453-456, 1962. A unit of activity is defined as the minimum quantity of ferment necessary to inactivate 1×10^{-7} M penicillin (60 units) in 1 hr at 37° C., pH 6.8-7.0.

TABLE 1

	Bacterial Cell Culture	β-Lactamase Activity (Units)
1	C-600 Su^+/pBR322-bla	833
2	C-600 Su^+/pBR322-bla + λcI_{857}	4400
3	C-600 Su^+/pBR322-bla + λ$cI_{857}Q_{am117}R_{am54}$	8300

[0050] As shown in Table 1, β-lactamase synthesis in the C-600 Su^+/pBR322-bla cells infected by phage λ with mutations in the later genes, Q and R, is almost 10 times greater than the synthesis in C-600 Su^+/pBR322-bla control cells.

EXAMPLE 2

Increased Output of β-Lactamase Encoded by bla-Gene Contained in Both Plasmid and Phage

[0051] Cultures of *E coli* W 3101 $recA^-13$ Su^- with and without transformation by pBR322-bla were cultivated in Aminopeptid medium diluted 1:1 with 0.15 M NaCl, at 37° C. to a density of 1×10^8 cells/ml. A $recA^-$ strain was used because these cells have a reduced ability to conduct recombination in areas of extended homology in both plasmid and phage (e.g. bla-gene). Thus, $recA^-$ cultures avoid exclusion of the homologous bla-gene. The cultures were divided into two portions. The first portion, which was not exposed to

phage, was incubated at 37° C. for 16 hr. The second portion was infected with phage λ $cI_{587}blaQ_{am\ 117}R_{am\ 54}$ at a multiplicity of about 10 phage bodies per 1 bacterial cell and cultivated for 2.5-3 hr at 37° C., and then for an additional 14 hr at 28° C. β-Lactamase activity was measured by the iodometric method. The results, shown in Table 2 (below), are expressed in units, as defined above for Table 1.

[0052] Phage λ $cI_{587}bla\ Q_{am\ 117}R_{am\ 54}$ was prepared from lysogenic cultures maintained at 28° C. in Aminopeptid medium. When the bacterial cell density reached about 1×10^8 cells/ml, the cells were warmed for 20 minutes at 43° C. in order to inactivate the cI repressor. Consequently, the prophage is excised from the bacterial genome and begins its lytic development. After 50 min, the cells underwent lysis, releasing 100-200 bodies each. At a density of 1×10^8 cells/ml, the cultures produced $1\text{-}2\times10^{10}$ phage bodies per ml. Thus, to infect bacterial cells with phage at a multiplicity of about 10 means that 1 ml of phage lysate (1×10^{10} phage bodies) was added to 10 ml of bacterial suspension (1×10^9 cells).

TABLE 2

	Bacterial Cell Culture	β-Lactamase Activity (Units)
1	W 3101 recA⁻13 Su°/pBR322-bla	13,555
2	W 3101 recA⁻13 Su° + $\lambda cI_{857}blaQ_{am117}R_{am54}$	227,796
3	W 3101 recA⁻13 Su°/pBR322-bla + $\lambda cI_{857}blaQ_{am117}R_{am54}$	2,000,000

[0053] As shown in Table 2, bacterial cells which were transformed with both plasmid containing the targeted gene and phage carrying the same gene, produced about 10 times more recombinant protein (β-lactamase) than bacterial cells transformed with phage alone, and over 100 times more β-lactamase than bacterial cells transformed by plasmid alone.

EXAMPLE 3

Super-Production of the β-Galactosidase Encoded by lac-Gene Contained in Both Plasmid and Phage

[0054] Cultures of *E. coli* RLM1 containing prophage λ cI_{857} plac5 $Q_{am\ 117}\ R_{am\ 54}$ (carrying a copy of the β-galactosidase gene, lac5) were grown in LB medium (Difco) at 30° C. with intensive aeration to a density of approximately 1×10^8 cells/ml. The lysogenic culture was warmed to 43° C. and incubated for 20 minutes to inactivate cI repressor. The temperature was then decreased to 37° C. and the bacterial cells underwent lysis, with phages being formed at $1\text{-}2\times10^{10}$ PFU/ml. Subsequently, 10 liters of phage lysate, containing about 1×10^{10} phage bodies (λ cI_{857} plac5 $Q_{am\ 117}\ R_{am\ 54}$) per ml, were added to 40 liters of a suspension of *E Coli* Ca 77 Su° transformed by plasmid pZ56 at a density of about 1×10^8 cells/ml in LB medium. Thus, the multiplicity of infection was 25, i.e., there were 25 phage bodies per bacterial cell.

[0055] After 7 hr at 37° C., recombinant β-galactosidase constituted 1.9 g per liter of culture medium. The activity of β-galactosidase was calculated by the method of Miller (Miller, J. H., EXPERIMENTS IN MOLECULAR GENETICS, 1972, Cold Spring Harbor Laboratory Press). A unit of activity was calculated as the minimum quantity of ferment required to hydrolyze 1 μM ortho-nitrophenyl-β-D-galactoside to orthonitrophenol per min at 30° C., pH 7.0.

EXAMPLE 4

Super-Expression of Human Interferon α-2b

[0056] Su^+ and Su° strains of *E. coli* K 802, transformed with a plasmid bearing a single copy of the gene encoding alpha-2 human interferon (pIF-2-trp), were grown in LB medium to a density of $1.5\text{-}2\times10^8$ cells/ml. These cells were then infected at a multiplicity of 15 with different phage λ lysates, as indicated in Table 3 (below). Cultivation continued with intensive aeration at 25° C. for 13 hr. Control cultures, not infected with phage, were incubated at 37° C. for the same period.

TABLE 3

	Bacterial Cell Culture	Phage	Interferon (Units/L)
1	K 802-pIF-2-trp Su^+		8.0×10^7
2	K 802-pIF-2-trp Su^+	λcI_{857}	77×10^7
3	K 802-pIF-2-trp Su^+	$\lambda cI_{857}Q_{am117}R_{am54}$	340×10^7
4	K 802-pIF-2-trp Su^+	λ-pIF-2-trp$cI_{857}Q_{am117}R_{am54}$	1400×10^7
5	K 802-pIF-2-trp Su°	λ-pIF-2-trp$cI_{857}Q_{am117}R_{am54}$	3000×10^7

[0057] As shown in Table 3, when bacterial cells which were transformed with plasmid containing the targeted gene were infected with phage λ containing only the temperature-sensitive mutation in cI, interferon expression increased by about 10-fold compared to control, non-infected cultures. Adding the amber-mutations in Q and R genes further increased expression by about 40-fold compared to control bacterial cells. Adding a copy of the interferon gene to phage λ with cI, Q and R mutations increased interferon synthesis by 175-fold over controls. Finally, when a non-suppressing, Su°, strain of *E. coli*, transformed by a plasmid bearing a copy of the interferon gene, was infected with phage λ, also having a copy of the interferon gene, as well as cI, Q and R mutations, the bacterial host cells produced about 375 times more recombinant protein than the control cells transformed by plasmid alone.

EXAMPLE 5

Enhanced Recovery of Biologically Active Recombinant Interferon by Phage-Mediated Host Cell Lysis

[0058] Strain *E. Coli* SG 20050 was transformed by a plasmid bearing two copies of the human interferon alpha-2b gene (pIF-14) by standard methods. The transformant cells were grown up in 80 liters of LB medium at 37° C. with intensive aeration to a density of 2×10^8 cells/ml. The culture was divided into two portions. The first was not infected with phage. The second was infected with phage λ lysate harvested from *E. coli* K 802/λ $cI_{857}\ Q_{am\ 117}\ R_{am\ 54}$ at a multiplicity of 10 phage bodies per bacterial cell. The control cells were incubated for 19 hr at 37° C. and the phage-infected cells were incubated for 19 hr at 21° C.

[0059] Interferon production in both control and phage-infected cultures was about 20% of the total cellular protein. However, the interferon in control cells was associated at least in part with insoluble inclusion bodies. Thus, it was not

possible to determine its biological activity without solubilization, denaturation and renaturation. In contrast, the specific activity of the soluble interferon released into the medium following phage-mediated cell lysis, was readily determined by standard immunoenzyme assay. The interferon activity was 4×10^{10} IU/liter (200 mg/liter).

[0060] Pre-clinical toxicological studies of recombinant human alpha-2β interferon produced by the phage superproduction method of the present invention showed that the compound was practically non-toxic. Intra-abdominal and intramuscular injections of the recombinant interferon in white mice and Wistar rats at 8.5×10^9 ME/kg (2.5×10^5 times the maximum human dose) and intravenous injections in mice and rabbits at 4.25×10^9 ME/kg (1.25×10^5 times the human therapeutic dose) produced no pronounced intoxication or death of the animals. Four months of injections in Wistar rats at 6×10^5, 6×10^6 and 3×10^7 ME/kg (18, 180 and 900 times the human therapeutic dose, respectively) showed no damage to the main organs and bodily systems of the experimental animals. Likewise, three months of intravenous injections in rabbits of 6×10^5 and 6×10^6 ME/kg, and two months of intramuscular injections in dogs at 3×10^6 ME/kg showed no signs of damage to the organ systems.

[0061] During immunotoxic and allergenic analysis of recombinant interferon, the induction of cellular and humoral immunity reactions, as well as delayed and immediate hypersensitivity reactions were studied. The results indicated that no immunotoxic or allergenic influence was produced. The recombinant interferon also had no mutagenic or DNA-damaging effects in bacteria during metabolic activation in vitro or in bone marrow of mouse embryos in vivo.

[0062] Embryotoxic studies of recombinant interferon were conducted in pregnant hamadryad baboon females. Daily intramuscular doses during organogenesis (20^{th} to 50^{th} days of pregnancy) caused defects in embryo development leading to miscarriage or stillbirth. Similar results were obtained for recombinant interferon analogs, and most probably could be explained by a powerful antiproliferative action of interferons. It is possible that the miscarriage may be attributed to a "cancellation" of immunologic tolerance of maternal organism towards the fetus, caused by immunomodulating action of the protein.

[0063] Recombinant interferon was also studied in Ukrainian clinics. Based on these clinical studies, the recombinant interferon was shown to be useful in the treatment of a variety of human diseases and conditions. For example, recombinant interferon was effective in treating acute and chronic hepatitis B, acute viral, bacterial and mixed infections, acute and chronic septic diseases, herpetic infections, herpes zoster, papillomatosis of larynx, multiple sclerosis, and various cancers, including melanoma, renal cell carcinoma, bladder carcinoma, ovarian carcinoma, breast cancer, Kaposi's sarcoma and myeloma.

[0064] The contraindications in human clinical applications were prolonged (several months) use at high doses, allergy and pregnancy. The possible side effects noted were small and transitory "flu-like" symptoms and at prolonged regimens, leuko and trombocytopenia were marked.

[0065] While we have described a number of embodiments of this invention, it is apparent that our description of the invention can be altered to provide other embodiments that utilize the basic process of this invention. Therefore, it will be appreciated by those of skill in the art that the scope of this invention is to be defined by the claims appended hereto rather than the specific embodiments that have been described in detail above by way of example.

What is claimed is:

1. A method for producing a biologically active protein, comprising:

transforming a bacterial host cell with a plasmid having at least one copy of an expressible gene encoding said protein;

infecting the transformed bacterial host cell with a bacteriophage capable of mediating lysis and also capable of lytic growth without lysis; and

cultivating the bacterial host cell under a culture condition that induces lytic growth of said cell without lysis until a desired level of production of said protein is reached.

2. The method of claim 1, wherein the bacteriophage has a temperature-sensitive mutation.

3. The method of claim 2, wherein the bacteriophage is bacteriophage λ and the temperature-sensitive mutation is cI_{857}.

4. The method of claim 2, wherein said culture condition that induces lytic growth of the bacteriophage is at a temperature of greater than 32° C.

5. The method of claim 2, wherein prior to the cultivating step, the bacterial host cells are grown at a temperature which prevents lytic growth of the bacteriophage.

6. The method of claim 5, wherein the temperature which prevents lytic growth of the bacteriophage is less than about 32° C.

7. The method of claim 1, wherein the bacteriophage has a mutation in at least one gene involved in bacteriophage-mediated lysis of the bacterial host cell.

8. The method of claim 7, wherein the bacteriophage is bacteriophage λ and the at least one gene involved in bacteriophage-mediated lysis is selected from the group consisting of N, Q and R.

9. The method of claim 1, wherein the bacterial host cell is a strain of *E. coli*.

10. The method of claim 9, wherein the strain of *E. coli* produces a suppressor for the repair of amber-mutations.

11. The method of claim 9, wherein the strain of *E. coli* lacks a suppressor for the repair of amber-mutations.

12. The method of claim 1, wherein the infecting bacteriophage is provided at a multiplicity of infection in a range of about 1 to about 100.

13. The method of claim 1, wherein the infecting bacteriophage is provided at a multiplicity of infection in a range of about 10 to about 25.

14. The method of claim 1, wherein bacteriophage-mediated lysis of the bacterial host cell is delayed at higher multiplicities of infection relative to lower multiplicities of infection.

15. The method of claim 1, wherein the bacteriophage contains at least one copy of an expressible gene encoding said protein.

16. A method for producing a biologically active protein, comprising:

transforming a bacterial host cell with a plasmid having at least one copy of an expressible gene encoding said protein;

infecting the transformed bacterial host cell with a bacteriophage having at least one copy of an expressible gene encoding said protein; and

cultivating the bacterial host cell under a culture condition that allows expression of said genes.

17. The method of claim 16, wherein the bacteriophage has a temperature-sensitive mutation.

18. The method of claim 17, wherein the bacteriophage is bacteriophage λ and the temperature-sensitive mutation is cI_{857}.

19. The method of claim 16, wherein the bacteriophage has a mutation in at least one gene involved in bacteriophage-mediated lysis of the bacterial host cell.

20. The method of claim 19, wherein the bacteriophage is bacteriophage λ and the at least one gene involved in bacteriophage-mediated lysis is selected from the group consisting of N, Q and R.

21. The method of claim 16, wherein the bacterial host cell is a strain of *E. coli*.

22. The method of claim 21, wherein the strain of *E. coli* produces a suppressor for repairing amber-mutations.

23. The method of claim 21, wherein the strain of *E. coli* lacks a suppressor for repairing amber-mutations.

24. A bacterial host cell with a plasmid having at least one copy of an expressible heterologous gene encoding a protein, wherein said host cell is infected with a bacteriophage capable of mediating lysis and also capable of lytic growth without lysis.

25. The bacterial host cell of claim 24, wherein the bacteriophage has a temperature-sensitive mutation.

26. The bacterial host cell of claim 25, wherein the bacteriophage is bacteriophage λ and the temperature-sensitive mutation is cI_{857}.

27. The bacterial host cell of claim 24, wherein the bacteriophage has a mutation in at least one gene involved in bacteriophage-mediated lysis of the host cell.

28. The bacterial host cell of claim 27, wherein the bacteriophage is bacteriophage λ and the at least one gene involved in bacteriophage mediated lysis is selected from the group consisting of N, Q and R.

29. The bacterial host cell of claim 24, wherein the bacteriophage is bacteriophage λ having cI_{857}, $Q_{am\ 117}$ and $R_{am\ 54}$ mutations.

30. The bacterial host cell of claim 24, wherein the bacteriophage has at least one copy of an expressible heterologous gene encoding said protein.

31. The bacterial host cell of claim 24, wherein the bacterial host cell is a strain of *E. coli*.

32. The bacterial host cell of claim 31, wherein the strain of *E. coli* lacks a suppressor for repairing amber-mutations.

33. The bacterial host cell of claim 31, wherein the strain of *E. coli* is recA deficient.

34. A strain of *E. coli* with a plasmid having at least one copy of an expressible heterologous gene encoding a protein, wherein said strain of *E. coli* is infected with bacteriophage λ having cI_{857}, $Q_{am\ 117}$ and $R_{am\ 54}$ mutations.

35. The strain of claim 34, wherein said protein is human alpha-2b interferon.

36. The strain of claim 34, wherein said strain of *E. coli* lacks a suppressor for repairing amber-mutations.

37. The strain of claim 36, further comprising recA-13.

38. A strain of *E. coli* with a plasmid having at least one copy of an expressible heterologous gene encoding a protein, wherein said strain of *E. coli* is infected with bacteriophage λ having cI_{857}, $Q_{am\ 117}$ and $R_{am\ 54}$ mutations and at least one copy of an expressible heterologous gene encoding said protein.

39. The strain of claim 38, wherein said strain of *E. coli* lacks a suppressor for repairing amber-mutations.

40. The strain of claim 37, wherein said protein is human alpha-2b interferon.

* * * * *

? b 155
01jul02 10:52:28 User208669 Session D2043.1
$0.32 0.090 DialUnits File1
$0.32 Estimated cost File1
$0.01 TELNET
$0.33 Estimated cost this search
$0.33 Estimated total session cost 0.090 DialUnits

File 155:MEDLINE(R) 1966-2002/Jun W5
*File 155: Daily alerts are now available. This file has been reloaded. Accession numbers have changed.

Set Items Description
--- ----- ----------
? ds
Set Items Description
S1 25982 LAMBDA
S2 22 DELAYED(W)LYSIS
S3 1 S1 AND S2
S4 23622 LYSIS
S5 245 S1 AND S4
S6 177992 DELAY?
S7 9 S5 AND S6
S8 562021 N
S9 26382 Q
S10 208764 R
S11 3014 S1 AND S8
S12 33 S4 AND S11
S13 11 S5 AND S9
S14 40 S5 AND S10
S15 72 S12 OR S13 OR S14
? t s3/7
3/7/1
DIALOG(R)File 155:MEDLINE(R)
08193955 94327465 PMID: 8051010
Functions involved in bacteriophage P2-induced host cell lysis and identification of a new tail gene.
Ziermann R; Bartlett B; Calendar R; Christie G E
Department of Molecular and Cell Biology, University of California, Berkeley 94720-3204.
Journal of bacteriology (UNITED STATES) Aug 1994, 176 (16) p4974-84, ISSN 0021-9193 Journal Code: 2985120R
Contract/Grant No.: AI-08722; AI; NIAID; GM-34651; GM; NIGMS
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

Successful completion of the bacteriophage P2 lytic cycle requires phage-induced lysis of its Escherichia coli host, a process that is poorly understood. Genetic analysis of lysis-deficient mutants defined a single locus, gene K, which lies within the largest late transcription unit of P2 and maps between head gene L and tail gene R. We determined and analyzed the DNA sequence of a ca. 2.1-kb EcoRV fragment that spans the entire region from L to R, thus completing the sequence of this operon. This region contains all of the functions necessary for host cell lysis. Sequence analysis revealed five open reading frames, initially designated orf19 through orf23. All of the existing lysis mutants--ts60, am12, am76, and am218--were located in orf21, which must therefore correspond to gene K. The K gene product has extensive amino acid sequence similarity to the product of gene R of bacteriophage lambda, and its exhibits endolysin function. Site-directed mutagenesis and reverse genetics were used to create P2 amber mutants in each of the four other newly identified open reading frames. Both orf19 (gene X) and orf20 (gene Y) encode essential functions, whereas orf22 (lysA) and orf23 (lysB) are nonessential. Gene Y encodes a polypeptide with striking similarities to the family of holin proteins exemplified by gpS of phage lambda, and the Yam mutant displayed the expected properties of a holin mutant. The gene products of lysA and lysB, although nonessential, appear to play a role in the correct timing of lysis, since a lysA amber mutant caused slightly accelerated lysis and a lysB amber mutant slightly delayed lysis of nonpermissive strains. Gene X must encode a tail protein, since lysates from nonpermissive cells infected with the X amber mutant were complemented in vitro by similar lysates of cells infected with P2 head mutants but not with tail mutants.
Record Date Created: 19940907
? t s7/7/3
7/7/3
DIALOG(R)File 155:MEDLINE(R)
08639170 95398948 PMID: 7669346
Holins: form and function in bacteriophage lysis.
Young R; Blasi U
Department of Biochemistry and Biophysics, Texas A&M University, College Station 77843, USA.
FEMS microbiology reviews (NETHERLANDS) Aug 1995, 17 (1-2) p191-205, ISSN 0168-6445 Journal Code: 8902526
Contract/Grant No.: GM27099; GM; NIGMS
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
During the lytic cycle of most bacteriophages, a phage-encoded peptidoglycan-degrading activity is elaborated. At least four entirely distinct types of enzymes fulfill this role and are given the generic name 'endolysin'. Endolysins characterized to date are synthesized without a signal sequence and thus accumulate fully folded and active in the cytosol

during the vegetative phase. Small membrane proteins are required in order for endolysins to gain access to the peptidoglycan. Because the available data suggest that the membrane lesion formed by these proteins is stable and non-specific, these proteins have been given the designation 'holins' ('hole'-formers). Analysis of the primary sequence suggests a simple membrane topology with two or more membrane-spanning helical domains and a highly charged, hydrophilic C-terminus. Comparison of the sequences of holins from phages of Gram-negative hosts suggests there are at least two major holin groups. Putative holin genes have also been found in bacteriophages of Gram-positive bacteria. Altogether, in phages of Eubacteria, 11 or more unrelated gene families which share the functional and structural characteristics of holins have been identified. Genetic and physiological analysis suggest that holins are primarily regulated at the level of function. Holin function is modulated in some cases by a second protein encoded by the holin gene. The primary regulation of holin function, however, appears to be intrinsic to the holin structure itself, since a missense allele of the S holin gene of phage lambda has been found which abolishes the normal delay that allows the vegetative phase to generate a useful number of progeny. (73 Refs.)
Record Date Created: 19951013
? t s15/7/9
15/7/9
DIALOG(R)File 155:MEDLINE(R)
10219927 99201027 PMID: 10099231
Characterization of bacteriophage lambda Q- mutant for stable and efficient production of recombinant protein in Escherichia coli system.
Lin C S; Chen B Y; Park T H; Lim H C
Department of Chemical and Biochemical Engineering and Materials Science, University of California, Irvine, California 92697-2575, USA.
Biotechnology and bioengineering (UNITED STATES) Mar 5 1998, 57 (5) p529-35, ISSN 0006-3592 Journal Code: 7502021
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
We previously demonstrated that the lambda system integrated into the host chromosome can overcome the instability encountered in continuous operations of unstable plasmid-based expression vectors. High stability of a cloned gene in a lysogenic state and a high copy number in a lytic state provide cloned-gene stability and overexpression in a two-stage continuous operation. But the expression by the commonly used S- mutant lambda was only twice as high as that of the single copy. To increase the expression in the lambda system, we constructed a Q- mutant lambda vector that can be used in long-term operations such as a two-stage continuous operation. The Q- mutant phage lambda is deficient in the synthesis of proteins involved in cell lysis and lambda DNA packaging, while the S- mutant is deficient in the synthesis of one of two phage proteins required for lysis of the host cell and liberation of the progeny phage. Therefore, it is expected that the replicated Q- lambda DNA containing a cloned gene would not be coated by a phage head and would remain naked for ample expression of the cloned gene and host cells would not lyse easily and consequently would produce larger amounts of cloned-gene products. The beta-galactosidase expression per unit cell by the Q- mutant in a lytic state was about 30 times higher than that in a lysogenic state, while the expression by the commonly used S- mutant in a lytic state was twice as high as that in a lysogenic state. The optimal switching time of the Q- mutant from the lysogenic state to the lytic state for the maximum production of beta-galactosidase was 5.3 h, which corresponds to an early log phase in the batch operation. Copyright 1998 John Wiley & Sons, Inc.
Record Date Created: 19990503
? t s15/7/22 23 24 29 30 33 34 38 44 46 60 63-65 68
15/7/22
DIALOG(R)File 155:MEDLINE(R)
09313828 97231470 PMID: 9102634
[The role of bacteriophage lambda coded proteins in destruction of the bacterial cell wall and the control of lysis timing]
Rola bialek kodowanych przez faga lambda w destrukcji bakteryjnej sciany komorkowej i kontroli czasu lizy.
Kedzierska S; Taylor A
Katedra Biochemii, Pracownia Biochemii Bakteryjnej, Uniwersytet Gdanski.
Postepy biochemii (POLAND) 1996, 42 (4) p331-9, ISSN 0032-5422 Journal Code: 0023525
Document type: Journal Article; Review; Review, Tutorial
Languages: POLISH
Main Citation Owner: NLM
Record type: Completed
(49 Refs.)
Record Date Created: 19970415

15/7/23
DIALOG(R)File 155:MEDLINE(R)
09149083 97032135 PMID: 8878031
Two beginnings for a single purpose: the dual-start holins in the regulation of phage lysis.
Blasi U; Young R
Institute of Microbiology and Genetics, Vienna Biocentre, University of Vienna, Austria. Udo@gem.univie.ac.at
Molecular microbiology (ENGLAND) Aug 1996, 21 (4) p675-82, ISSN 0950-382X Journal Code: 8712028
Contract/Grant No.: GM27099; GM; NIGMS
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

For most large phages of both Gram-positive and Gram-negative bacteria, there appears to be a single pathway for achieving disruption of the host envelope, requiring at least two phage-encoded lysis functions (a holin and an endolysin). The holin is a small membrane protein which causes a non-specific lesion in the cytoplasmic membrane, which allows the endolysin to gain access to its substrate, the peptidoglycan. The scheduling of host lysis is effected by regulatory mechanisms which govern the synthesis and activity of the holin protein accumulating in the membrane. Accordingly, aspects of expression and function of holin genes are considered here, focusing mainly on the lambdoid S genes. This group of genes, of which lambda S is the prototype, are characterized by a dual-start motif consisting of two Met start codons separated by one or two codons, at least one of which specifies Arg or Lys. Two protein products are elaborated, differing only by two or three N-terminal residues but apparently possessing opposing functions: the shorter polypeptide is the active holin, or lysiseffector, whereas the longer polypeptide apparently acts as an inhibitor of holin function. Models will be considered which may account for the ability of the holin to form a 'hole' in the cytoplasmic membrane at a programmed time, as well as for the inhibitory properties of the longer product. Finally, we discuss recent results suggesting that the dual-start motif can be viewed as a level of regulation superimposed on a timing function intrinsic to the canonical holin structure. (39 Refs.)
Record Date Created: 19970127

15/7/24
DIALOG(R)File 155:MEDLINE(R)
09061279 96417847 PMID: 8820638
The two-step lysis system of pneumococcal bacteriophage EJ-1 is functional in gram-negative bacteria: triggering of the major pneumococcal autolysin in Escherichia coli.
Diaz E; Munthali M; Lunsdorf H; Holtje J V; Timmis K N
Department of Microbiology, GBF-National Research Centre for Biotechnology, Braunschweig, Germany.
Molecular microbiology (ENGLAND) Feb 1996, 19 (4) p667-81, ISSN 0950-382X Journal Code: 8712028
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The holin function Ejh of the pneumococcal bacteriophage EJ-1 has been characterized. It shows structural features similar to, and functionally complemented, the prototype member of the holin family. In Escherichia coli and Pseudomonas putida the Ejh product caused cellular death, and changes in cell morphology could be accounted for by lesions in the cytoplasmic membrane. Expression of ejh resulted in the inhibition of growth in a variety of phylogenetically distant bacterial genera, suggesting a broad spectrum of action. Concomitant expression of the ejh and ejl (encodes a lysin) genes led to lysis of E. coli and P. putida cells. Remarkably, the Ejl lysin was able to attack murein from bacteria lacking choline in their sacculi, which suggests that pneumococcal lysins have a broader substrate specificity than previously assumed. Furthermore, the Ejh holin was able to trigger activity of the major pneumococcal autolysin cloned and expressed in E. coli, and this raised new questions about the regulation of this model autolysin. A new function for holins in systems where the phage lysin is supposed to be associated with the membrane is proposed.
Record Date Created: 19961216

15/7/29
DIALOG(R)File 155:MEDLINE(R)
08576981 95336699 PMID: 7612243
Temperature induction of bacteriophage lambda mutants in Escherichia coli.
Chen B Y; Lin C S; Lim H C
Department of Chemical and Biochemical Engineering, School of Engineering, University of California, Irvine 92717, USA.
Journal of biotechnology (NETHERLANDS) Jun 1 1995, 40 (2) p87-97, ISSN 0168-1656 Journal Code: 8411927
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The paper presents temperature induction in Escherichia coli cells with phage lambda on the target-protein production and cell growth. Replicated lambda-DNA particles in the Q- and S- mutants remain naked for a longer time by preventing DNA packaging and cell lysis, and therefore the expression of the foreign genes is high. However, the parasitic infection of phage-lambda causes on significant losses of host cell viability in the induction phase. The temperature effects on cell growth and targeted-gene product formation were investigated. Gene amplification was found to be growth phase dependent for both Qam73 (Q mutation) and Sam100 (S mutation) mutants. Maximum induction occurs in the early exponential phase and under the optimal cell density. The total beta-galactosidase activity at this optimal induction condition increases roughly 8-10-fold with respect to that without thermal induction. To maximize the induction efficiency for the gene-product beta-galactosidase activity, several operating parameters were investigated. In this study, temperature induction is strongly dependent upon the population density of 'susceptible' cells at which time the temperature is shifted to 38-42 degrees C. This may be due to the 'threshold' population density to regulate the infection of lambda to hosts and control the productivity of target gene expression.
Record Date Created: 19950824

15/7/30
DIALOG(R)File 155:MEDLINE(R)

08529971 95286513 PMID: 7768829
S gene expression and the timing of lysis by bacteriophage lambda.
Chang C Y; Nam K; Young R
Department of Biology, Texas A&M University, College Station 77843-2128, USA.
Journal of bacteriology (UNITED STATES) Jun 1995, 177 (11) p3283-94, ISSN 0021-9193 Journal Code: 2985120R
Contract/Grant No.: 27099; PHS
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The S gene of bacteriophage lambda encodes the holin required for release of the R endolysin at the onset of phage-induced host lysis. S is the promoter-proximal gene on the single lambda late transcript and spans 107 codons. S has a novel translational initiation region with dual start codons, resulting in the production of two protein products, S105 and S107. Although differing only by the Met-1-Lys-2... N-terminal extension present on S107, the two proteins are thought to have opposing functions, with the shorter polypeptide acting as the lysis effector and the longer one acting as an inhibitor. The expression of wild-type and mutant alleles of the holin gene has been assessed quantitatively with respect to the scheduling of lysis. S mRNA accumulates during the late gene expression period to a final level of about 170 molecules per cell and is maintained at that level for at least the last 15 min before lysis. Total S protein synthesis, partitioned at about 2:1 in favor of the S105 protein compared with the other product, S107, accumulates to a final level of approximately 4,600 molecules per cell. The kinetics of accumulation of S is consistent with a constant translational rate of less than one S protein per mRNA per minute. Mutant alleles with alterations in the translational initiation region were studied to determine how the translational initiation region of S achieves the proper partition of initiation events at the two S start codons and how the synthesis of S105 and S107 relates to lysis timing. The results are discussed in terms of a model for the pathway by which the 30S ribosome-fMet-tRNA complex binds to the translational initiation region of S. In addition, analysis of the relationship between lysis timing and the levels of the two S gene products suggests that S107 inhibits S105, the lethal lysis effector, by a stoichiometric titration.
Record Date Created: 19950706

15/7/33
DIALOG(R)File 155:MEDLINE(R)
08331068 95089686 PMID: 7997166
A dominant mutation in the bacteriophage lambda S gene causes premature lysis and an absolute defective plating phenotype.
Johnson-Boaz R; Chang C Y; Young R
Department of Biochemistry and Biophysics, Texas A&M University, College Station 77843.
Molecular microbiology (ENGLAND) Aug 1994, 13 (3) p495-504, ISSN 0950-382X Journal Code: 8712028
Contract/Grant No.: GM27099; GM; NIGMS
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The S and R genes of the bacteriophage lambda are required for lysis of the host. R encodes 'endolysin', a soluble transglycosylase which accumulates in the cytoplasm during late protein synthesis. S encodes a 'holin', a small membrane protein which, at a precisely scheduled time, terminates the vegetative cycle by forming a lethal lesion in the membrane through which gpR gains access to the peptidoglycan. A missense allele of S, Ala52Gly, causes lysis to occur prematurely at about 19-20 min after induction of a lysogen, compared to 45 min for the wild type. This allele has a severe plaque-forming defect which appears to be entirely a consequence of the early lysis and resultant severe reduction in particle burst size. The early-lysis phenotype is dominant and is aggravated, in terms of an even more reduced burst size, at both 30 degrees C and 42 degrees C. The mutation maps in the middle of a putative membrane-spanning helical domain of S, near the sites of other S- mutations with recessive non-lytic phenotypes. The mutation has no effect on S-protein accumulation or on the ratio of S107 and S105 products in the membrane. The mutation appears to affect the intrinsic timing function by which the S protein controls the lysis schedule.
Record Date Created: 19950117

15/7/34
DIALOG(R)File 155:MEDLINE(R)
08292313 95050320 PMID: 7961508
Inducible cell lysis system for the study of natural transformation and environmental fate of DNA released by cell death.
Kloos D U; Stratz M; Guttler A; Steffan R J; Timmis K N
Department of Microbiology, National Research Centre for Biotechnology (GBF), Braunschweig, Germany.
Journal of bacteriology (UNITED STATES) Dec 1994, 176 (23) p7352-61, ISSN 0021-9193 Journal Code: 2985120R
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Two novel conditional broad-host-range cell lysis systems have been developed for the study of natural transformation in bacteria and the environmental fate of DNA released by cell death. Plasmid pDKL02 consists of lysis genes S, R, and Rz from bacteriophage lambda under the control of the Ptac promoter. The addition of inducer to Escherichia coli,

Acinetobacter calcoaceticus, or Pseudomonas stutzeri containing plasmid pDKL02 resulted in cell lysis coincident with the release of high amounts of nucleic acids into the surrounding medium. The utility of this lysis system for the study of natural transformation with DNA released from lysed cells was assessed with differentially marked but otherwise isogenic donor-recipient pairs of P. stutzeri JM300 and A. calcoaceticus BD4. Transformation frequencies obtained with lysis-released DNA and DNA purified by conventional methods and assessed by the use of antibiotic resistance (P. stutzeri) or amino acid prototrophy (A. calcoaceticus) for markers were comparable. A second cell lysis plasmid, pDKL01, contains the lysis gene E from bacteriophage phi X174 and causes lysis of E. coli and P. stutzeri bacteria by activating cellular autolysins. Whereas DNA released from pDKL02-containing bacteria persists in the culture broth for days, that from induced pDKL01-containing bacteria is degraded immediately after release. The lysis system involving pDKL02 is thus useful for the study of both the fate of DNA released naturally into the environment by dead cells and gene transfer by natural transformation in the environment in that biochemically unmanipulated DNA containing defined sequences and coding for selective phenotypes can be released into a selected environment at a specific time point. This will allow kinetic measurements that will answer some of the current ecological questions about the fate and biological potential of environmental DNA to be made.
Record Date Created: 19941227

15/7/38
DIALOG(R)File 155:MEDLINE(R)
07699174 93223998 PMID: 8467992
Non-specific hole formation in the Escherichia coli inner membrane by lambda S proteins in independent of cellular secY and secA functions and of the proportion of membrane acidic phospholipids.
Rietsch A; Blasi U
Institute of Microbiology and Genetics, University of Vienna, Austria.
FEMS microbiology letters (NETHERLANDS) Feb 15 1993, 107 (1) p101-5, ISSN 0378-1097 Journal Code: 7705721
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Formation of the lesion in the Escherichia coli inner membrane caused by lambda lysis protein S was examined by electron microscopy. We also show that macromolecules exceeding the size of the lambda R transglycosylase can pass through the S-dependent hole and that assembly of the S-dependent hole is independent of the proportion of acidic phospholipids in the inner membrane and of components of the cellular transport machinery.
Record Date Created: 19930511

15/7/44
DIALOG(R)File 155:MEDLINE(R)
07048877 91354716 PMID: 1367360
Secretion of active bovine somatotropin in Escherichia coli.
Klein B K; Hill S R; Devine C S; Rowold E; Smith C E; Galosy S; Olins P O
Monsanto Corporate Research, Monsanto Co., St. Louis, MO 63198.
Bio/technology (Nature Publishing Company) (UNITED STATES) Sep 1991, 9 (9) p869-72, ISSN 0733-222X Journal Code: 8309273
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
We have expressed a chimeric protein, comprising the LamB secretion signal sequence fused to mature bovine somatotropin (bST), in Escherichia coli. Plasmid constructs with the recA promoter showed significant protein accumulation prior to induction and cell lysis occurred after induction. In contrast, the lacUV5 promoter was tightly regulated. With the lacUV5 promoter, temperature and inducer concentration had significant effects on the total amount of recombinant protein produced and the fraction processed to mature bST. Quantitation of bST from shake flask cultures showed that 1-2 micrograms/ml/OD550 could be released from the periplasm by osmotic shock. N-terminal sequence analysis of the purified protein indicated that the majority of the secreted bST was correctly processed. The bST present in the osmotic shock fraction was judged to be correctly folded by comigration with oxidized methionyl-bST standard on a non-reducing polyacrylamide gel and activity in a bovine liver radioreceptor assay. These results provide a rapid method to produce bST for use in structure-function studies.
Record Date Created: 19911010

15/7/46
DIALOG(R)File 155:MEDLINE(R)
06826161 91150530 PMID: 2291440
Intracellular lytic enzyme systems and their use for disruption of Escherichia coli.
Dabora R L; Cooney C L
Merck and Co., Inc., Elkton, VA 22827.
Advances in biochemical engineering/biotechnology (GERMANY) 1990, 43 p11-30, ISSN 0724-6145 Journal Code: 8307733
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
This article focusses on lytic enzyme systems available in E. coli and their potential use for cellular disruption. In the systems described here the genetic information for lysis would be carried within the microbial host, either integrated or naturally occurring on chromosomal DNA, or on extrachromosomal elements such as plasmids. Each microbe would carry

complete information for endogenous enzymatic lysis, and lysis would occur in a controlled manner after being triggered by an external factor such as temperature or inducer addition. The lytic systems explored in this review include the autolytic enzymes, colicin lytic enzymes, and bacteriophage lytic enzymes from phage phiX174, T4, lambda, MS2 and Q beta. Many of the colicin lytic enzymes and all of the bacteriophage lytic enzymes described here have been cloned, and in some instances examined as cellular disruption methods. None of the E. coli autolytic enzymes have been cloned, but information pertinent for use as a disruption method is described. (95 Refs.)
Record Date Created: 19910401

15/7/60
DIALOG(R)File 155:MEDLINE(R)
04503968 84185450 PMID: 6232260
Facile and gentle method for quantitative lysis of Escherichia coli and Salmonella typhimurium.
Crabtree S; Cronan J E
Journal of bacteriology (UNITED STATES) Apr 1984, 158 (1) p354-6, ISSN 0021-9193 Journal Code: 2985120R
Contract/Grant No.: AI 15650; AI; NIAID
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Garrett et al. (Mol. Gen. Genet. 182:326-331, 1981) constructed strains of Escherichia coli harboring derivatives of plasmid pBR322 that carry the lysis genes (S, R, and Rz) of phage lambda. The plasmid construction placed the genes under control of the lactose operon operator-promotor (and thus of lac repressor). Induction of E. coli strains carrying these plasmids resulted in rapid lysis of the culture unless the S gene was defective, in which case the cells grew normally. A freeze-thaw treatment of induced cells carrying an S- plasmid gave quantitative lysis of either E. coli or Salmonella typhimurium cells under exceptionally gentle conditions. The method was equally effective on exponential phase cells and stationary phase cells and was readily extended to a large number of independent cultures.
Record Date Created: 19840607

15/7/63
DIALOG(R)File 155:MEDLINE(R)
03992537 82265503 PMID: 6213604
Effect of the lambda S gene product on properties of the Escherichia coli inner membrane.
Wilson D B
Journal of bacteriology (UNITED STATES) Sep 1982, 151 (3) p1403-10, ISSN 0021-9193 Journal Code: 2985120R
Contract/Grant No.: GM25030; GM; NIGMS
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The S gene of bacteriophage lambda is a late gene required for cell lysis, but unlike the other two lysis genes, R and Rz, it does not code for an endolysin. Earlier studies have shown that the S gene product inhibits respiration and macromolecular synthesis and makes the inner membrane permeable to sucrose. In this study, the effect of the S gene product on a number of Escherichia coli membrane functions (active transport, permeability, respiration, and transhydrogenase and ATPase activity) were measured, and a product of the lambda S gene was identified in the inner membrane fraction by two-dimensional polyacrylamide gel electrophoresis. The results of these experiments indicate that the lambda S product is present in the inner membrane, that it increased the permeability of the membrane for all of the small molecules that were tested, and that its action is reversible. The simplest explanation of these results is that the S gene product forms a hydrophilic pore through the inner membrane, allowing small molecules and lambda lysozyme to pass through.
Record Date Created: 19821029

15/7/64
DIALOG(R)File 155:MEDLINE(R)
03770356 82035239 PMID: 6457237
Cell lysis by induction of cloned lambda lysis genes.
Garrett J; Fusselman R; Hise J; Chiou L; Smith-Grillo D; Schulz J; Young R
Molecular & general genetics : MGG (GERMANY, WEST) 1981, 182 (2) p326-31, ISSN 0026-8925 Journal Code: 0125036
Contract/Grant No.: GM27099-01; GM; NIGMS
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The lysis gene region of bacteriophage lambda, including genes S, R, and Rz, was cloned into the plasmid pBH20. In the recombinant plasmid, the lysis genes are expressed under the control of the lacOP region. Induction of this "lysis operon" with the lac inducer, IPTG, under conditions where transcription from the lacOP region is not subject to catabolite repression, results in a sharply defined lysis after 35 min. Premature lysis can be accomplished by cyanide, chloramphenicol, or chloroform, exactly as in bacteriophage lambda infected cells. The lysis gene region of an S- mutant was also cloned into pBH20. Induction of the S- lysis operon has no apparent effect on culture growth; however, large quantities of bacteriolytic activity accumulate intracellularly. Neither cyanide nor chloramphenicol causes lysis in the induced S- clones. Thus premature lysis

appears to be entirely an S-dependent phenomenon. A model for the control of lysis in bacteriophage lambda infections is presented in which it is the accumulation of the S gene product in competition with a host "anti-S" protein that determines lysis timing.
Record Date Created: 19811215

15/7/65
DIALOG(R)File 155:MEDLINE(R)
03658031 81210186 PMID: 6453650
Protein degradation in E. coli: the Ion mutation and bacteriophage lambda N and cII protein stability.
Gottesman S; Gottesman M; Shaw J E; Pearson M L
Cell (UNITED STATES) Apr 1981, 24 (1) p225-33, ISSN 0092-8674 Journal Code: 0413066
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The Ion gene of E. coli controls the stability of two bacteriophage lambda proteins. The functional half-life of the phage N gene product, measured by complementation, is increased about 5-fold in Ion mutant strains, from 2 min to 10 min. The chemical half-life of N protein, determined by its disappearance on polyacrylamide gels following pulse-chase labeling, increases about three-fold in Ion cells. In contrast to its effect on the N protein, the Ion mutation produces a 50% decrease in the chemical half-life of cII protein. The decay rate of many other phage proteins, including the unstable gene O product, remains unaffected by a host Ion defect. A Ion mutation alters lambda physiology in two ways. First, upon infection, the phage enters the lytic pathway predominantly. This may result from the deficiency of cII protein caused by its decreased stability, since cII product is required for establishment of lysogeny. Second, brief thermal induction of a Ion (lambda c1857) lysogen leads irreversibly to lysis; repression cannot be restablished and the treated cells are committed to forming infective centers. Although N product is normally required for rapid commitment, Ion lysogens become committed more rapidly than Ion+ lysogens, even in the absence of N function. These results identify for the first time native proteins whose stability is affected by the Lon proteolytic pathway. They also indicate that the Lon system may be important in regulating gene expression in E. coli.
Record Date Created: 19810810

15/7/68
DIALOG(R)File 155:MEDLINE(R)
03352111 80164825 PMID: 6245342
The construction in vitro of derivatives of bacteriophage lambda carrying the amidase genes of Pseudomonas aeruginosa.
Drew R E; Clarke P H; Brammar W J
Molecular & general genetics : MGG (GERMANY, WEST) Jan 1980, 177 (2) p311-20, ISSN 0026-8925 Journal Code: 0125036
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The amidase genes of Pseudomonas aeruginosa were inserted into a lambda replacement vector following cleavage with the restriction endonuclease HindIII. The recombinant lambdaami was detected by enhanced growth of Escherichia coli around plaques of the recombinant phage on minimal medium containing acetamide as the nitrogen source. Low levels of amidase activity were detected in E. coli cultures infected with lambdaami and these were sufficient to allow growth with acetamide as nitrogen source. Lysis-defective derivatives of lambdaami were made by introducing Q-, S-, mutations. Cultures of E. coli infected with lambdaamiQ-S- synthesised amidase as the major protein. The amidase produced by these cultures was identical to that produced by PAC strains of P. aeruginosa in substrate specificty, thermal stability and immunological cross-reaction.
Record Date Created: 19800616
? save temp
Temp SearchSave "TD744" stored
? log hold
01jul02 11:10:12 User208669 Session D2043.2
$6.39 1.996 DialUnits File155
$0.00 81 Type(s) in Format 6
$3.78 18 Type(s) in Format 7
$3.78 99 Types
$10.17 Estimated cost File155
$3.90 TELNET
$14.07 Estimated cost this search
$14.40 Estimated total session cost 2.087 DialUnits
Logoff: level 02.05.22 D 11:10:12

File

United States Patent [19]

Auerbach et al.

[11] **Patent Number: 4,637,980**

[45] **Date of Patent: Jan. 20, 1987**

[54] **EXTERNALIZATION OF PRODUCTS OF BACTERIA**

[75] Inventors: **Jeffrey I. Auerbach**, King of Prussia; **Martin Rosenberg**, Malvern, both of Pa.

[73] Assignee: **SmithKline Beckman Corporation**, Pa.

[21] Appl. No.: **521,517**

[22] Filed: **Aug. 9, 1983**

[51] **Int. Cl.**[4] **C07H 15/12**; C12P 21/00; C12N 15/00; C12N 1/00; C12N 1/20

[52] **U.S. Cl.** **435/68**; 435/172.3; 435/172.1; 435/317; 435/253; 935/22; 935/32; 935/33; 935/38; 935/47; 536/27

[58] **Field of Search** 435/172.3, 317, 68; 424/93; 935/22, 32, 33, 38, 47; 536/27

[56] **References Cited**

FOREIGN PATENT DOCUMENTS

74808	9/1981	European Pat. Off. .	
61250	9/1982	European Pat. Off. .	
2084584	4/1982	United Kingdom	435/172.3

OTHER PUBLICATIONS

Maniatis et al, 1982, *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory, pp. 17-27.

Kleckner, 1979, (Abstract) "DNA Sequence Analysis of Tn10 Insertions: Origin and Role of 9 bp Flanking Repetions During Tn10 Translocation", Cell V 16(u) 711-20.

Rosenberg et al., Methods in Enzymology 101:123-138, (1983).

Kleckner et al., J. Mol. Biol. 116:125-159 (1977).

Remaut et al., *Gene* 15:81 (1981).

Sninsky et al., *Gene* 16:275 (1981).

Primary Examiner—Thomas G. Wiseman
Assistant Examiner—Joanne M. Giesser
Attorney, Agent, or Firm—Edward T. Lentz; Janice E. Williams; Alan D. Lourie

[57] **ABSTRACT**

A bacterial product is made by transforming a temperature sensitive lysogen with a DNA molecule which codes, directly or indirectly, for the product, culturing the transformant under permissive conditions and externalizing the product by raising the temperature to induce phage encoded functions.

31 Claims, No Drawings

EXTERNALIZATION OF PRODUCTS OF BACTERIA

FIELD OF THE INVENTION

This invention relates to genetic engineering and, in particular, to externalization of products produced by genetically engineered microorganisms.

BACKGROUND INFORMATION

A problem with using *E. coli* and other prokaryotic microorganisms as hosts for expression of desired proteins has often been externalizing the proteins from the host cells for purification. Attempts to overcome this problem include physical disruption of cells such as by homogenization or sonication, chemical disruption of cells such as by treatment with detergent or lysozyme, and fusing a DNA sequence which codes for an excretion signal peptide to a structural gene coding for the desired product. For example, Weissman et al., European Patent Application No. 61,250, disclose treatment of host cells with a lysing or permeabilizing agent; Silhavy et al., U.S. Pat. No. 4,336,336, disclose a method for fusing a gene for a cytoplasmic protein to a gene for a non-cytoplasmic protein, so that a resulting hybrid protein is transported to, near or beyond the host cell surface; Gilbert et al., U.S. Pat. No. 4,338,397, disclose a method for producing mature secreted proteins comprising inserting a structural gene for a preprotein into an expression vector.

E. coli can be infected by an obligatory parasite, the lambda phage, which is a double-stranded DNA virus. Lambda genetics, like *E. coli* genetics, is well-studied. See, for example, "The Bacteriophage Lambda," edit. by A. D. Hershey, Cold Spring Harbor Laboratory, New York, 1971.

Lambda, a temperate phage, multiplies in *E. coli* in either of two phases. In one, the lytic phase, the phage DNA replicates autonomously and directs formation of capsid proteins, packaging and host cell lysis. Expression of lambda DNA during the lytic phase is highly efficient. Transcription occurs on both DNA strands, on one in the rightward direction and on the other in the leftward direction. Induction can result in release of about one hundred phage particles within 50 minutes at 37° C. See, Hershey, above.

In the other phase, the lysogenic phase, lambda DNA is integrated into the host cell genome and is replicated, passively, along with the host chromosomal DNA by the host replication enzymes. A phage in the lysogenic phase is known as a prophage; the host is known as a lysogen and is said to be immune.

Immunity can be lost by occurrence of various events which induce the lytic phase. The products of the lambda int and xis genes catalyse excision of the lambda genome from the *E. coli* genome to form a covalently closed circle capable of autonomous replication. The synthesis of these genes, and either directly or indirectly, all other lambda genes is repressed by the product of the lambda cI gene. In response to certain chemicals or DNA-damaging agents, the bacteria directs synthesis of the product of the bacterial recA gene. The recA gene product proteolytically cleaves the cI repressor protein, permitting expression of the lytic phase genes. Propagation of the phage then requires interplay of several lambda regulatory elements which ultimately initiate autonomous replication of the lambda DNA. The products of the lambda P and O genes are required for DNA replication. Subsequent to DNA replication the phage must direct synthesis of viral structural proteins, that is, head and tail proteins, and their assembly into intact empty virions. Interaction of at least 18 genes is required to accomplish this. Finally, the DNA is packaged into the empty virions to produce infectious intact virions, and the cell is ruptured by endolysin, coded for by the lambda S and R genes which are activated by the product of the Q gene, thereby releasing the phages. The Q gene is activated by the N function. The N gene is repressed by the cI function.

The 18 genes required for capsid assembly lie between about map positions 3 and 36 on the rightward transcription strand, map positions being representative of percentages of total lambda DNA. The first genes, from left to right, are A, W and B; the last is J. In normal lysogens, to the right of the J gene are eight bacterial genes. Five of these, bio A, B, C, D and F, are involved in biosynthesis of biotin. A sixth, uvrB, confers resistance to ultraviolet radiation. The final two, chlA and E, confer sensitivity to chlorates. See, Guest, *Mol. Gen. Genet.* 105: 285–289 (1969) and Stevens et al, in "The Bacteriophage Lambda", ed. by Hershey, et al, cited above, at pp. 515–534.

Another lambda gene which functions in natural host cell lysis is the kil gene. The function of the kil gene is not fully understood. Cells which express the kil gene have a decreased rate of cell growth following induction. Loss of the kil function permits cells to grow at normal rates, that is, log phase growth, after induction, until lysis occurs. Like the S and R genes, the kil gene is regulated by the cI repressor, indirectly, through the N gene. See, Greer, *Virology* 66:589–604 (1975).

Temperature sensitive lysogens have been well-studied. They are described, for example, by Campbell, *Virology* 14: 22–32 (1961). The cI857 gene is a temperature sensitive cI mutant. It is functional at or below 38° C. See, Sussman et al., *C.R.H. Acad. Sci. Paris* 254:1517–1519 (1962). Similar phage systems are known to occur in other genera. For example, Lomovskaya et al., *J. Virol.* 9:258–262 (1972), report temperature sensitive mutants of a temperate phage which infects Streptomyces; Flock, *Mol. Gen. Genet.* 155: 241–247 (1977), reports temperature sensitive mutants of the temperate phage, phi-105, which infects Bacillus; Botstein et al., *Nature* 251: 584–588 (1974) report temperature sensitive mutants of the temperate phage, P22, which infects Salmonella. Jostrom et al., *J. Bacteriol.* 119:19–32 (1974), and Thompson, *J. Bacteriol.* 129:778–788 (1977), report temperature sensitive mutants of the temperate phage, phi-11, which infects Staphylococcus; Miller et al, *Virol.* 59:566–569 (1974) report temperate phages of Pseudomonas.

The lambda endolysin has been found to lyse Salmonella strains which are able to absorb the phage as reported by Botstein et al., *Ann. Rev. Genetics* 16:61–83 (1982).

Perricaudet et al., *FEBS Lett.* 56:7–11 (1975), describe deletion of lambda genes between map positions 58 and 71 (Δ 58–71) which segment includes the lambda int, xis, red, gam, cIII and kil genes.

Hershberger et al., United Kingdom Specification Application No. 2,084,584, disclose use of a lysogen as a host cell to stabilize and select for the presence of a plasmid. The authors disclose, for example, transforming a lysogen having a defective cI gene with a plasmid

carrying a functional cI gene. In one disclosed embodiment, the functional cI gene is the cI857 gene.

It is known that transposable elements, that is, genes which can recombine independently of host chromosomal recombination mechanisms, can be inserted into host cells as markers. Ross et al., *Cell* 16:721-731 (1979), report physical structures of deletions and inversions promoted by the transposable tetracycline-resistance element, tn10. Davis et al., "Bacterial Genetics", Cold Spring Harbor Laboratory, New York (1980), describe uses of transposable elements.

Ruvkun et al., *Nature* 289:85-88 (1981), report integration of the transposable kanamycin resistance and neomycin resistance element, tn5, into *Rhizobium meliloti* chromosomal DNA by conjugation of a plasmid carrying tn5 followed by homologous recombination. Integration of a heterologous gene by recombination resulting from presence of homologous flanking sequences is also disclosed in European Patent Application No. 74,808.

SUMMARY OF THE INVENTION

The invention is a method of producing a product in bacteria utilizing endolysin-encoding genes from temperate phages. The method comprises transforming a temperature sensitive bacterial strain, which carries a temperature sensitive phage repressor gene and functional phage lysozyme-encoding genes such that the lysozyme-encoding genes are repressed under permissive conditions and expressed under restrictive conditions, with a DNA molecule(s) which expresses, directly or indirectly, the product; culturing the transformed strain under permissive conditions such that the product is made; raising the temperature to produce restrictive conditions; and, optionally, recovering the product from the culture medium or a concentrate thereof.

Another aspect of the invention is a method of producing a product in a bacteria which comprises transforming a bacteria which produces the product with a phage DNA sequence which carries a temperature sensitive phage repressor gene and phage lysozyme-encoding genes such that the lysozyme-encoding genes are repressed under permissive conditions and expressed under restrictive conditions, to make the bacteria lytic; culturing the transformed bacteria under permissive conditions such that the product is made; changing the temperature to provide restrictive conditions; and, optionally, recovering the product from the culture medium or a concentrate thereof.

Another aspect of the invention is a DNA fragment comprising a defective phage sequence having a temperature sensitive repressor gene and functional lysozyme-encoding genes such that the lysozyme-encoding genes are repressed under permissive conditions and expressed under restrictive conditions, a selectable marker and, preferably, flanking DNA sequences which are homologous to a contiguous sequence in the chromosome of a host cell.

Other aspects of the invention are a method of making a lytic bacteria which comprises transforming a bacteria with the DNA fragment of the invention, and bacteria comprising said DNA fragment.

Yet another aspect of the invention is a method of administering a product to a mammal comprising administering an amount of a temperature sensitive bacteria containing an effective dose of the product to the mammal, whereby the bacteria lyse within the mammal and release the product.

DETAILED DESCRIPTION OF THE INVENTION

A temperature sensitive bacteria is one which carries a prophage DNA sequence including a temperature sensitive repressor gene such that when cultured at one temperature range (permissive conditions) the repressor is functional but when cultured at another temperature range (restrictive conditions) the repressor is not functional; the repressor is not expressed or is not stable. Under restrictive conditions, the phage genes, including phage lysozyme-encoding genes, are expressed leading to cell lysis. Such temperature sensitive bacteria are lytic bacteria. Any lytic bacteria, as herein defined, can be used in the method of the invention.

Temperature sensitive temperate phage repressor genes are available or can be made by mutating such genes by procedures known to the art. By way of example, Campbell, *Virology* 14:22-32 (1961), describes a procedure for isolating temperature sensitive phage mutants. Generally, the procedure comprises mutagenizing phage-infected bacteria, such as by ultra-violet irradiation, and then incubating survivors at high temperature to cause induction of any temperature sensitive repressor mutants. The lysate is then used to infect sensitive bacteria. The new lysogens are subjected to heat induction and phage produced following the heat induction are used to prepare lysogens from sensitive bacteria. This cycling (lysogen preparation, induction, re-preparation) leads to identification of phage repressor mutants. Typically, 3 to 4 such cycles are sufficient to yield such mutants.

The description which follows relates, in large part, to lytic *E. coli* and, especially, to cI857 *E. coli* lysogens. Nevertheless, from said description persons of ordinary skill in the art will be enabled to practice the invention as it relates to other lytic *E. coli* as well as to other lytic bacteria, using repressor and endolysin-encoding genes from lambda or from other temperate phages, such as the temperate phages noted above.

cI857 lysogens, which are known and commonly available, produce cI repressor which is active at or below 38° C. but inactive above 38° C. These are preferred over other temperature sensitive *E. coli* lysogens because in addition to a mutation rendering the repressor inactive above 38° C., the cI857 gene contains a second mutation which causes the cI repressor protein to be insensitive to proteolytic cleavage by the product of the lambda recA gene. Thus, when cultured under permissive conditions, the cI repressor protein is stable and, therefore, effective in maintaining immunity.

E. coli strain UC5822 is a lysogen which has the cI857 mutation. It also has a point mutation in the int gene (int 6 am, an amber mutation) and in the P gene (P3 am, an amber mutation). (Amber mutations signal termination of translation). UC5822 is generally preferred over, for example, MM294(cI857) because UC5822 is defective, that is, it does not generally produce infectious phage particles. Defective lysogens are preferred, especially when used to administer a polypeptide to a mammal. UC5822, however, produces lower levels of the lysozyme, presumably because it has a lower copy number of the S and R genes, namely, one, than does MM294(cI857), namely, fifty to one hundred, after induction. Nevertheless, UC5822 lyses readily following induction.

In one aspect of the method of the invention, temperature sensitive bacteria are transformed with a DNA mmolecule(s) which codes, directly or indirectly, for a desired product. The transformation may be carried out by any technique which allows the DNA molecule to enter the host cell and to express the product. Techniques include, for example, transformation, transduction, conjugation and cell fusion. Many suitable expression vectors are well known and publicly available as are techniques for cloning genes for products and transforming cells with such molecules. Generally, the product will be a non-excreted, heterologous gene product, that is, one which is not naturally produced by the host and which is not externalized. Products which are expressed directly include polypeptides; products which are expressed indirectly include polypeptides, glycoproteins, antibiotics and other molecules such as, for example, metal ions which can be sequestered within a metallothionein-producing bacteria.

Transformed host cI857 lysogens can be grown up indefinitely under permissive conditions (≦38° C., usually 32° to 36° C.) which are optimal for expression of the desired product. When sufficient growth has been achieved, that is, usually, when mid-log phase growth (A_{650}=0.5) has been achieved, synthesis of lambda endolysin is induced by culturing the lysogen under restrictive conditions. This can be accomplished by raising the temperature of the culture medium, or of a cell concentrate thereof, to, in the case of cI857, greater than 38° C., preferably 42° to 44° C., for about 90 to 120 minutes. Alternatively, the temperature of the culture medium, or of a concentrate thereof, is raised to greater than 38° C., preferably 42° to 44° C., for a shorter time, that is, a time sufficient to induce the phage DNA, preferably at least about five minutes, following which the temperature is lowered to 0° to 38° C., preferably 2° to 36° C.

Maintaining restrictive conditions for 90 to 120 minutes is preferred because lysis is more efficient and rapid. However, the latter procedure is preferred in certain applications, for example, when a desired protein is heat labile or when the cost of maintaining the restrictive conditions is prohibitive.

If the host cI857 lysogen has a functional lambda cro gene, the cells will continue to synthesize the lysozyme at any temperature at which the cells function, until lysis occurs. Although lambda endolysin is active as low as 0° C., the time needed for lysis is longer at low temperature due to a decrease in rates of protein synthesis and catalytic activity generally.

Just prior to or following induction, the cells are preferably concentrated, such as by filtration, centrifugation or other means, and incubated in this concentrated form until lysis. Such procedure facilitates collection and purification of the desired product. Following induction, the bacterial cell wall is substantially degraded. The cells, in the form of protoplasts, will continue to synthesize the desired product which is largely released into the medium through the cell membranes. Complete release into the medium is effected by lysis. Lysis is observable as a clarification of the culture medium or concentrate and/or an increase in the viscosity of the culture medium or concentrate. Lysis can be enhanced such as by mechanical agitation or rapidly changing the culturing conditions, for example, by rapidly changing temperature between 2° and 25° C. or changing the osmotic strength of the medium or concentrate. Preferably, after concentrating cells and decanting product-containing supernatant, induced cells are suspended in a minimal salts buffer or 0.1M tris buffer, 50 mM NaCl and 1 mM EDTA and agitated to effect lysis.

The desired product can then be recovered from the medium or concentrate and purified, if desirable, by known techniques.

In an alternative procedure, whole cells are concentrated and administered orally to a mammal prior to induction of the lytic phase. Induction will then occur internally, resulting in release of the desired polypeptide. This method can be especially useful for administering antigens to animals in cases in which whole cells as well as the desired antigen are preferred to provoke an immunoprotective response. For example, temperature sensitive lysogens carrying genes which code for antigens such as the LT-B antigen can be fed directly to pigs and/or calves. The amount of cells administered to each animal will be that amount which contains an effective dose. The amount of protein produced by a unit amount of cells can be calculated by known techniques.

An aspect of the invention is a DNA fragment which can be used to construct a lytic bacteria for use in the method of the invention. Such DNA fragment comprises a defective phage sequence having a temperature sensitive repressor gene and functional lysozyme-encoding genes. Such DNA fragment comprising a defective lambda sequence has a temperature sensitive cI gene and functional lambda endolysin-encoding genes (N, Q, S and R) such that the endolysin is expressed under restrictive conditions, a selectable marker, and, preferably, flanking DNA sequences which are homologous to a contiguous DNA sequence in a host cell chromosome. In one particular embodiment, the DNA fragment comprises lambda DNA which is deleted in the genes lying between map positions 58 and 71, and therefore lacks the int, xis and kil genes, has a temperature sensitive cI gene and has mutations in the O and P genes and in which the cI gene is the cI857 mutant and produces endolysin under restrictive conditions. The O and P mutations can be deletion or point mutations. Point mutations, such as the O29, P3 and P80 mutations, are preferred because they are readily available. The P3 mutation is preferred over the P80 mutation.

In another particular embodiment, the fragment comprises lambda DNA which is substantially deleted in the genes lying between map positions 3 and 71. Such fragment lacks substantially all genes essential for lambda capsid assembly as well as the int, xis and kil genes.

A host cell, *E. coli* or other bacteria, which produces a desired product, or which is previously or subsequently made to produce the desired product, such as by genetic engineering techniques, can be transformed with a phage DNA sequence which carries temperature sensitive phage repressor gene and phage lysozyme-encoding genes by known techniques. These include infecting the bacteria with a temperate phage having such temperature sensitive repressor gene, preferably a defective phage. These also include transforming the bacteria with the DNA fragment of the invention by known techniques, for example, transformation, transduction, conjugation and fusion. Transformation generally involves incorporating the fragment into a vector, such as a phage or a plasmid. For example, the fragment can be cloned into a plasmid, such as pBR322 or others, and grown up in vivo in an appropriate a host which is

lacking a contiguous sequence homologous to sequences flanking the fragment or which is defective for recombination events (rec−). The plasmid can be recovered and used to transform an appropriate host for production of a desired product. Following transformation of such host, the fragment which has flanking DNA sequences homologous to a contiguous DNA sequence in the host chromosome will integrate by spontaneously recombining at the site of the homologous contiguous sequence. Alternatively, an appropriate host for production of a desired product can be transformed with the isolated DNA fragment in linear or circular form.

The DNA fragment carries a selection marker to facilitate selection of transformants. Selectable markers are typically genes which code for assayable enzymes, which restore prototrophy to an auxotrophic host or which confer resistance to lethal or inhibitory compounds, usually antibiotics. Preferably, the selection marker is a gene which confers antibiotic resistance as these do not require use of an auxotrophic host which may not be available or which can spontaneously revert to prototrophy. Tetracycline resistance is preferred because tetracycline is inexpensive and because resistance to tetracycline is not normally spontaneously acquired.

Presence of the marker in transformants indicates that the host comprises the DNA fragment. If the fragment integrates, the whole fragment will integrate because homology between the DNA fragment and the host cell DNA exists only in regions flanking the lambda DNA and the marker.

Absent a marker in the DNA fragment, selection of host cells carrying the lambda DNA following transduction or other transforming procedure would require super-infecting putative transductants with a defective phage (non-integrating) and selecting for immune bacterial survivors.

E. coli strains made lytic by integration of a DNA fragment of the invention include, for example, MG strains. These strains are lysogens in which the lambda DNA is deleted in genes lying between map positions 58 and 71, and therefore lack the int, xis, red, gam, kil and cIII genes, has the cI857 mutation and has mutations in the O and P genes and has functional N, Q, S and R genes such that endolysin is expressed under restrictive conditions. In one embodiment, strain MG1[C600 (λ Δ 58–71, cI857, P3, O29), SuII+, galK, lacZ, thi, gal::tn10 tet^R], the point (amber) mutation, is read through and the O and P genes are expressed because of the production by the host cell DNA of an amber suppressor, that is, a translational suppressor of the UAG translation termination codon.

A more preferred host cell for use in the method of the invention is one which is phenotypically O− and P−. One embodiment, strain MG3 [N99 (λ Δ 58–71, cI857, P3, O29) galK, lacZ, thi, gal::tn10 tet^R], carries the same lambda DNA fragment as strain MG1. However, it is phenotypically O− and P− as well as tet^R, Δ kil, Δ int and Δ xis.

The most preferred lytic *E. coli* are MG4 strains. These are strains which are deleted in substantially all of the lambda structural protein and assembly genes and the normal right prophage-bacterial junction, that is, the right attachment site (att^R). In particular, they are lysogens which are deleted in substantially all lambda genes lying between map positions 3 and 71, have point mutations in the O and P genes, have a temperature sensitive cI gene and have functional N, Q, S and R genes such that endolysin is expressed under restrictive conditions,and have a selectable marker, namely, the tn10 tetracycline resistance transposable element. Such defective lysogens have 4 independent blocks to viral propagation: (i) loss of O and P replication functions, (ii) loss of att^R which renders the prophage incapable of being complemented by int and xis genes from a superinfecting phage, (iii) inability to encode lambda structural genes and, (iv) the size of the lambda DNA is far below the minimum size required for packaging. These can be initially prepared by chlorate-stressing cI857, O−, P− lysogenic strains, such as MG strains, to produce chlorate resistant mutants and selecting such mutants which are unable to complement propagation of a superinfecting heteroimmune or virulent lambda or lambdoid phage deficient in A and B functions. A DNA fragment comprising the marker, the lambda DNA and flanking sequences from the *E. coli* chromosome can be isolated from MG4 strains such as by treatment with restriction endonucleases or P1 transduction.

MG4 can be derived from MG3 by deleting all or most of the bacteriophage genes which encode the viral structural components. In order to verify the loss of these genes it is sufficient to demonstrate that the viral genome in MG4 is unable to complement and propagate a superinfecting phage which is itself defective for these genes. λ charon 3A (λ A−, B− immϕ80) is an example of a phage which can be used for this superinfection. Alternatively any phage carrying amber mutations in the A, B or other viral structural cistron can be plated on sensitive *E. coli* in the presence of a heteroimmune or virulent phage (λ vir). Recombination will occur between the two phages and lead to the formation of a recombinant, for example, λ virA−. The frequency of recombinants will be between 1–50%, depending on the experimental conditions. Recombinants can be recognized by their ability to plate on suppressor containing lysogens [Y mel (λ)] and their inabililty to produce plaques on non-suppressing, λ sensitive strains, such as N99. Plaques obtained from the above cross are plated onto petri dishes containing Y mel (λ) or N99 and recombinants are purified. Lambda phages deficient in A, and/or B gene function are preferred since as a consequence of their position on the lambda genome MG4 candidates which cannot complement for these functions must lack all other lambda structural genes. The use of defective phages and hosts in this way is referred to as "marker rescue" and is widely practiced, See, for example, "The Bacteriophage Lambda," edit by A. D. Hershey, Cold Spring Harbor Laboratory, 1971, especially, Stevens et al., at pp. 515–533.

The instant invention can be used to produce any product of bacteria. Examples are many and include, among others, insulin, rabies glycoprotein, K99 and 987P antigens, antibiotics, growth hormones, metallothioneins, alpha-1-antitrypsin, influenza antigens, lymphokines and interferon. In addition, the invention can be used in colony screening, RNA isolation and plasmid preparation, as the invention greatly simplifies and shortens the time needed for such procedures by bypassing the lysis step otherwise required.

In the following examples, which are illustrative of the invention and not limiting, all starting materials are readily available or can be readily prepared by techniques known in the art. Transductions were carried out substantially as described in "Experiments in Molecular Genetics", edit. by J. H. Miller, Cold Spring Harbor

Laboratory, New York, (1972) pp. 201–205, which is incorporated herein by reference as though fully set forth.

EXAMPLE 1

Construction of MGO

Strain C600 (*E. coli* SuII+ K12 gall lacZ suII thi) was incubated in the presence of λ cI857 P3 O29 (gift of W. Syzbalski, U. of Wisconsin). After overnight growth at 32° C., surviving bacteria were isolated and purified. Eighty percent of these bacteria were found to be immune to superinfection, to be unable to grow at 44° C., and to produce lambda phage (following exposure to 44° C.) which were indistinguishable from λ cI857 P3 O29 (as judged by the ability of the phage to produce plaques on strain C600 but not on strain N99 (*E. coli* K12 galK lacZ suO thi). One of this class of survivors was purified and given the designation MGO (C600 (λ cI857 P3 O29)).

EXAMPLE 2

Construction of MGO-AR6

Strain N5151 (*E. coli* K12 SA500 galK lacZ pro thr his gal8 (λ cI857 Δ 58–71 Δ H1)) was incubated in the presence of Plcm100 phage which had been grown on strain AR4 (*E. coli* K12 gal::tn10 (Plcm100)). The cross between N5151 and Plcm100 grown on AR4 resulted in the isolation of tetracycline resistant, UV sensitive, temperature sensitive lysogens. One of these isolates was purified and designated MGO-AR6 (*E. coli* K12 gal8 gal::tn10 λΔ 58–71 cI857 Δ H1(bio uvrB)).

EXAMPLE 3

Construction of MG1

MGO-AR6 was made a Plcm100 lysogen by isolating survivors of AR6 which had been incubated in the presence of Plcm100. The Plcm100 lysogen of MGO-AR6 was designated MGO-AR18.

Strain MGO was crossed by P1 transduction with Plcm100 which had grown on MGO-AR18. After permitting time for phage absorption, the cells were subjected to a UV fluence of 4 J/m^2 (irradiation of 254 nm light was at a rate of 2 J/m^2/s as determined by a UV dosimeter) and incubated in the presence of tetracycline. Eleven percent of tetracycline resistant colonies were resistant to UV light indicating that they did not carry the H1 deletion and thus that they possessed the lambda genes from cI through the right hand end of the phage. Specifically, this means that these clones carry the P3 and O29 mutations and intact S and R genes. One third of the UV resistant, tetracycline resistant cells were incapable of producing phage. These were therefore judged to have acquired the 58–71 deletion of lambda, and thus to have lost the int, xis and kil genes. This class was purified and designated MG1 (C600) (λΔ 58–71 cI857 P3 O29) SuII+ galK lacZ thi gal::tn10 tet^R).

EXAMPLE 4

Construction of MG3

MG1 was incubated in the presence of Plcm100 and survivors were purified. Among these survivors, a high percentage of MG1 cells which had become Plcm100 lysogens were identified. A Plcm100 lysogen of MG1 was purified and designated MG2.

Strain N99 was crossed by Plcm100 transduction with Plcm100 which had grown on MG2. Tetracycline resistant transductants were selected. All of these were found to be immune to lambda and were therefore judged to be lambda lysogens. One of these lysogens was purified and designated MG3 (N99 (λΔ 58–71 cI857 P3 O29)).

MG3 was determined to lyse subsequent to exposure to 44° C. for 90–120 minutes. No phage were found in cell cultures either prior to or after such exposure (<1/0.1 ml of a culture having 3.3×10^8 cells per ml). The presence of phage was assayed on C600 cells. Control cultures of *E. coli* strains which harbor non-defective lambda prophages contained between 10^5 and 10^9 phages per ml of a culture having 3.3×10^8 cells per ml.

EXAMPLE 5

Construction of MG3

Strain MG3 was constructed substantially as described in the above Examples except that strain N99 was lysogenized directly with λ cI857 P3 O29. The resulting lysogen was crossed by P1 transduction with strain MGO-AR18 and tetracycline resistant lysogens which lysed upon temperature induction but which did not produce phage were selected.

EXAMPLE 6

Construction of UC5822

Strain UC5822 was constructed by infecting strain N99 with λ int6 red3 cI857 P80 and λ hy5 cIimm21Δ b2. λ hy5 cIimm21 Δ b2 is a hybrid between phage λ and phage 21. The purpose of λ hy5 cIimm21 Δ b2 in this construction is to provide int function in trans which is required in order for λ int6 red3 cI857 P80 to lysogenize this strain. The Δ b2 mutation renders the Δ hy5 cIimm21 Δ b2 phage incapable of directing its own integration into this strain. A survivor of this cross which displayed immunity to superinfecting lambda but was sensitive to phage 21 was purified and designated UC5822. The strain does not survive exposure to 44° C. No phage could be detected in cultures of UC5822 either before or after incubation at 44° C.

EXAMPLE 7

Construction of MG4

Cultures of MG3 are grown in Luria broth or other complete media at 32° C. until A_{650}=0.5. The culture will contain approximately 5×10^8 cells/ml. The culture is then plated on Nutrient Agar plates supplemented with 0.2% glucose, and 0.2% $KClO_3$. The plates are incubated under anerobic conditions at 32° C. until colonies form (3–5 days. Growth on this media under these conditions selects for *E. coli* which have mutations in the chl A, B, C or D gene. See, "Expts. in Molecular Genetics", J. Miller, pps 226–227.

Mutation in chlB, C or D will not lead to the isolation of MG4. Among the mutations affecting chlA expression will be point mutations in chlA and deletions extending to the left or right of chlA. Deletions extending to the left of chlA may result in disruption of the adjacent uvrB gene and thus confer a UV sensitive phenotype on the organism. For the same reason, rightward extending deletions from the chlD gene may also confer a UV sensitive phenotype on the organism. Chlorate resistant colonies obtained from the anerobic incubation are tested to determine if they are now UV sensitive. This is conveniently done by streaking a chlorate resistant colony across a petri dish, covering ½ the dish and

subjecting the other half to 10 J/m² of 254 nm UV light. This dose is sufficient to kill UV sensitive cells but not UV resistant mutants. The UV sensitive mutants (comprising mutations in chlA or chlD) are tested for the presence of the defective lambda prophage. This is done by cross streaking the cells through a streak of a homoimmune phage. Lambda sensitive bacteria are killed by the phage at the cross-streak; lambda lysogens are immune to superinfection and are not killed. As a consequence of the location of the chlA and chlD genes, the lamba genome and the uvrB gene, all UV sensitive chlD mutants will be lambda sensitive whereas some UV sensitive chlA mutants may be lambda lysogens. UV sensitive, lambda lysogens therefore contain deletions of chlA which extend leftward into uvr B. If the deletion extends through uvrB it may extend into the biotin operon and possibly into the structural lambda genes. Deletions of structural lambda genes have been obtained in this manner (Grier, *Virology* 66:589–604 (1975). All UV sensitive, chlA⁻, lambda lysogens are infected with λ virA⁻. After 2½ hours, the lysates are plated for λ virA⁻ phage and for λ vir A⁺ recombinants. MG4 candidates which propagate λ virA⁻ and/or produce λ virA⁺ phages are discarded; candidates which fail to complement λ virA⁻ or produce virA⁺ carry a deletion extending from chlA through the A gene of lambda. Those MG4 candidates which possess deletions from chlA through the A gene of lambda are tested for the lytic bacteria property (lysis upon growth at >38° C.). Candidates containing the deletion which have retained the lytic bacteria property are purified as MG4.

EXAMPLE 8

Cloning in MM294(cI857) and UC5822

E. coli strain MM294 was incubated in the presence of λ cI857. After overnight growth at 32° C., surviving bacteria were isolated and purified. Clones which were immune to superinfection and which were unable to grow and produced phage at 44° C. were isolated. This resulting cI857 lysogenic strain, MM294 (λ cI857), and *E. coli* strain UC5822 were made competent by $CaCl_2$ treatment and transformed with pDN5, a plasmid carrying genes for the *E. coli* LT-B antigen and for ampicillin and tetracyline resistance.

Ampicillin and tetracycline resistant transformants of both lytic bacteria strains grew well in standard nutrient broth at 30°–32° C. and expressed LT-B antigen. The bacteria were pelleted by centrifugation and transferred to a standard nutrient broth at 42° C. Within about 90–120 minutes, cell lysis was evident and substantially complete. LT-B antigen was released into the broth. In a sample of the MM294(cI857) transformant comprising 4×10^7 cells/ml, about 2×10^8 lambda phage were collected per ml. In a similar sample of the UC5822 transformants, no phage (<20/ml were collected.

EXAMPLE 9

Cloning in UC5822

A seed culture of *E. coli* uC5822 containing the plasmid pESS2 which carries the genes for *E. coli* LT-B antigen was inoculated in a 5 ml tube of L broth containing ampicillin. After 6 hours the tube contents were transferred to 500 ml of culture medium containing ampicillin and incubated overnight with shaking at 32° C. To inoculate 10 L, 400 ml of the overnight culture was transferred to 10 L of medium containing ampicillin in a Virtis bench-top fermentor. The culture was maintained at 32° C. and each hour a 100 ml sample was monitored for growth at A_{420}. At 4 hours the culture was fed 200 ml of 50% dextrose and 0.1 ml of an antifoam agent. At 8 hr the culture was at 4.8 A_{420} units and was shifted to 43° C. The culture was fed again at 10 hr with 200 ml of 50% dextrose, and at 14 hr the culture was stopped. A sample of a cell concentrate ("pellet") and of the cell supernatant at 4 hr, 6 hr, 8 hr, 9 hr, 10 hr, 10.6 hr and 14 hr were tested for LT_B by using an ELISA test with known concentrations of LT_B as standards.

The results are shown in Table I. In the first 4 to 8 hr greater than 90% of the LT-B resided in the cell, but within 2 hr after the temperature shift (10 hr after inoculation, 90% of the LT-B was in the supernatant. At 6 hr after the temperature shift, 95% of the LT-B was in the supernatant; LT-B represented 8.5% of the total protein. The yield of LT-B was far greater than the yield from *E. coli* MM294 transformed with peSS2.

Within 2–4 hours after the temperature shift lysis was evident by increased viscosity of the culture media and visible cell debris. By 4–6 hours after the temperature shift the viscosity was greatly reduced and the culture was easily pumped through an ultrafiltration apparatus to remove all debris and any remaining unlysed cells. The increased viscosity reflects release of high molecular weight DNA and RNA into the media; action of endogenous nucleases ultimately results in an observable decrease in viscosity.

TABLE I

	Time After Inoculation			A_{420}	mg/ml Cell Protein	μg/ml LT_B	Total LT_B As Percent of Total Cell Protein
(Shift)	4	hr	Pellet	0.58	0.734	0.41	
(43°)	4	hr	Super			0.06	0.07%
	6	hr	Pellet			1.1	
	6	hr	Super	1.8	1.19	.11	0.1%
	8	hr	Pellet	4.8	1.24	3.9	
	8	hr	Super			0.29	0.3%
	10	hr	Pellet	6.2	0.646	1.2	
	10	hr	Super			14.8	2.5%
	10.6	hr	Pellet	5.0	0.652	1.8	
	10.6	hr	Super			39.38	6.3%
	14	hr	Pellet			1.8	
	14	hr	Super	4.2	0.652	53.83	8.5%

EXAMPLE 10

Construction of Lytic Salmonella

An interspecies cross between Salmonella and MG3 is performed, either through conjugation or DNA transformation. Salmonella strains are normally tetracycline sensitive; MG3 is tetracycline resistant. Salmonella recombinants which have attained resistance to tetracycline are tested for their ability to grow at 42° C. Those Salmonella which lyse at this temperature have acquired, through recombination, the lytic bacteria function of MG3. This experiment is possible because (1) the lambda lytic functions are expressed in Salmonella and (2) sufficient homology exists between Salmonella and *E. coli* (MG3) to permit recombination of the *E. coli* sequences which flank the genes into Salmonella.

EXAMPLE 11

Construction of Lytic Bacillus

Method 1. The genetic elements sufficient to direct lysis of a host include the λ cI857, N, Q, S and R genes and the P_L promoter. The restriction maps of these

genes is known (Molecular Cloning, Maniatis et al., Cold Spring Harbor Laboratory, N.Y.). The genes are subcloned from lambda onto a plasmid (for example pBR322). A fragment of DNA from Bacillus is inserted into the plasmid at a site in a non-essential region. It is not necessary to characterize the nature or function of the host strain DNA or its orientation in the plasmid. The Bacillus is then incubated with the purified plasmid DNA, and antibiotic resistant transformants are selected. These transformants are tested to determine if they lyse after exposure to high temperature. In these transformants, the plasmid containing the lambda genes is present as an autonomously replicating unit or is integrated into the host chromosome through a recombination event between homologous Bacillus DNA on the plasmid and on the chromosome. The integration of DNA carried by plasmids which cannot replicate into the Bacillus chromosome has been described (Haldenway et al., J. Bact. 142:90–98, 1980). This method requires the expression of the lambda lytic genes in the recipient host, but does not require homology between the *E. coli* sequences of MG3 and the recipient bacteria.

Method 2. The phage phi-105 infects Bacillus, is temperate, and has a mutatable cI-like repressor. Derivatives of phi-105 can be made which have temperature sensitive mutations affecting this repression. Phage derivatives which lack excision or replication functions can be isolated through mutagenesis or by isolation of deletion strains. (Flock, *Mol. Gen. Genet.* 155:241-247, 1977). A phage derivative is obtained which possesses a thermo-labile repressor. A lysogen of this mutant is made by infecting sensitive cells with the phage at 30° C., isolating surviving cells and testing these cells for immunity to superinfecting phage and for inability to grow at 40° C. Such an organism is a phage-producing lytic bacteria. To isolate a defective lytic bacteria, the bacteria is mutagenized and surviving colonies are replica plated onto an undeveloped lawn of phi-105 sensitive Bacillus. Temperature-sensitive colonies which, following exposure to high temperature, produce few or no phage on these lawns, contain mutations affecting phage propagation. More stringent mutants may be obtained by repeating the mutagenesis. In order to mobilize this construction and easily select for transfer of this construction it is preferable to isolate a derivative which harbours an antibiotic resistance marker linked to the phi-105 genome. This can be accomplished by cloning random fragments of the Bacillus chromosome into a plasmid (which is incapable of replication in Bacillus) which carries an antibiotic resistance determinant (such as pBR322). Transformed, drug resistant cells are isolated which contain the integrated plasmid. In some of these cells the plasmid will have integrated near to the site of phi-105. The unfractionated pool of drug resistant colonies is infected with the generalized Bacillus transducing phage, pBS1. Stock of pBS1, which functions in Bacillus in exactly the same manner as Plcm100 functions in *E. coli*, is used to transduce Bacillus cells to drug resistance. These drug resistant transductants are tested to determine if they are thermosensitive, lytic bacteria. Approximately 1% of transductants will have acquired the lytic bacteria properties.

The preceding disclosure and examples show that the methods and compositions of matter of the invention are useful to produce and externalize products in bacteria. While the preferred embodiments of the invention are illustrated by the above, the invention is not limited to the precise constructions disclosed herein but, rather, includes all embodiments and modifications coming within the scope of the following claims.

We claim:

1. A method of producing a gene product which comprises (i) culturing a temperature sensitive bacteria, which bacteria:
   (a) expresses the gene product intracellularly,
   (b) is a lysogen defective in excision and replication functions and
   (c) contains within the prophage DNA sequence a temperature sensitive phage repressor gene and functional phage lysozyme-encoding genes, under permissive conditions such that the gene product is expressed intracellularly and the lysozyme-encoding genes are repressed and then (ii) raising the temperature to produce restrictive conditions such that the lysozyme-encoding genes are expressed.

2. The method of claim 1 wherein the temperature-sensitive phage repressor gene is a temperature sensitive lambda cI gene.

3. The method of claim 2 wherein the cI gene is the cI857 mutant.

4. The method of claim 2 wherein the bacteria is an *E. coli* lambda lysogen.

5. The method of claim 4 wherein the bacteria is *E. coli* strain UC5822.

6. The method of claim 4 wherein the lambda prophage DNA sequence is deleted in the genes lying between map positions 58 and 71 and is mutated in the O and P genes resulting in loss of O and P gene functions.

7. The method of claim 6 wherein the lambda prophage DNA sequence is flanked by a selectable marker which is a gene coding for a selectable trait and the O and P mutations are point mutations.

8. The method of claim 7 wherein the lambda prophage DNA sequence is flanked on the upstream end by the tn10 transposable tetracycline resistance element and the O and P genes are the O29 and P3 genes.

9. The method of claim 7 wherein the bacteria is an *E. coli* MG strain.

10. The method of claim 7 wherein the bacteria is strain MG3.

11. The method of claim 6 wherein the lambda Prophage DNA sequence is deleted in the genes lying between map positions 3 and 71.

12. The method of claim 11 wherein the lambda prophage DNA sequence is flanked by a selectable marker which is a gene coding for a selectable trait and the O and P mutations are point mutations.

13. The method of claim 12 wherein the lambda prophage DNA sequence is flanked on the upstream end by the tn10 transposable tetracycline resistance element and the O and P genes are the O29 and P3 genes.

14. The method of claim 12 wherein the bacteria is an *E. coli* MG4 strain.

15. A DNA fragment comprising (i) a selectable marker which is a gene coding for a selectable trait; (ii) a lambda prophage DNA sequence having a temperature sensitive cI repressor gene and functional lysozyme encoding genes such that the lysozyme-encoding genes are repressed under permissive conditions and expressed under restrictive conditions, wherein the prophage DNA sequence includes functional N, Q, S and R genes, is substantially deleted in the genes lying between map positions 58 and 71 and has mutations in the O and P genes resulting in loss of O and P gene functions; and, (iii) flanking DNA sequences homologous to a contiguous sequence in the chromosome of a host

bacterial cell to permit recombination between the fragment and the host cell chromosome to occur.

16. The DNA fragment of claim 15 wherein the cI gene is the cI857 gene, the O and P mutants are the O29 and P3 mutants and the selectable marker is the tn10 transposable tetracycline resistance element.

17. The DNA fragment of claim 15 wherein the prophage DNA sequence is substantially deleted in the genes lying between map positions 3 and 71.

18. The DNA fragment of claim 17 wherein the cI gene is the cI857 gene, the O and P mutants are the O29 and P3 mutants and the selectable marker is the tn10 transposable tetracycline resistance element.

19. A bacteria comprising the DNA fragment of claim 15.

20. A bacteria comprising the DNA fragment of claim 17.

21. The bacteria of claim 19 which is an *E. coli.*

22. The bacteria of claim 20 which is an *E. coli.*

23. The bacteria of claim 21 which is an MG strain.

24. The bacteria of claim 21 which is strain MG3.

25. The bacteria of claim 22 which is strain MG4.

26. A method of making a temperature sensitive bacteria which comprises transforming a bacteria with a DNA fragment comprising (i) a selectable marker which is a gene coding for a selectable trait; (ii) a lambda prophage DNA sequence having a temperature sensitive cI repressor gene and functional lysozyme encoding genes such that the lysozyme-encoding genes are repressed under permissive conditions and expressed under restrictive conditions, wherein the prophage DNA sequence includes functional N, Q, S and R genes, is substantially deleted in the genes lying between map positions 58 and 71 and has mutations in the O and P genes resulting in loss of O and P gene functions; and, (iii) flanking DNA sequences homologous to a contiguous sequence in the chromosome of a host bacterial cell to permit recombination between the fragment and the host cell chromosome to occur.

27. The method of claim 26 wherein the cI gene is the cI857 gene, the O and P mutants are the O29 and P3 mutants and the selectable marker is the tn10 transposable tetracycline resistance element.

28. The method of claim 26 wherein the prophage DNA sequence is substantially deleted in the genes lying between map positions 3 and 71.

29. The method of claim 28 wherein the cI gene is the cI857 gene, the O and P mutants are the O29 and P3 mutants and the selectable marker is the tn10 transposable tetracycline resistance element.

30. The method of claim 26 wherein the bacteria is an *E. coli.*

31. The method of claim 28 wherein the bacteria is an *E. coli.*

* * * * *

US005196318A

United States Patent [19]

Baldwin et al.

[11] Patent Number: 5,196,318

[45] Date of Patent: Mar. 23, 1993

[54] **PRECISELY REGULATED EXPRESSION OF DELETERIOUS GENES**

[75] Inventors: **Thomas O. Baldwin**, Bryan; **Jerry H. Devine**; **Gerald S. Shadel**, both of College Station, all of Tex.

[73] Assignee: **The Texas A&M University System**, College Station, Tex.

[21] Appl. No.: **544,268**

[22] Filed: **Jun. 26, 1990**

[51] **Int. Cl.**[5] **C12P 21/00**; C12N 15/09; C12N 15/70; C12N 15/31

[52] **U.S. Cl.** **435/69.1**; 435/172.3; 435/320.1; 536/27

[58] **Field of Search** 435/320.1, 172.3, 69.1; 536/27

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,503,142	3/1985	Berman et al.	435/6
4,578,355	3/1986	Rosenberg	435/320.1
4,634,678	1/1987	Salstrom et al.	435/320.1
4,677,064	6/1987	Mark et al.	435/69.1
4,767,708	8/1988	Minkley, Jr. et al.	435/194
4,775,630	10/1988	Tibbetts et al.	435/320.1
4,782,022	11/1988	Puhler et al.	435/172.3

OTHER PUBLICATIONS

Raab, et al. (Sep. 1986), "Mutational analysis of bacteriophage lambda lysis gene S", J. Bacteriol. 167, 1035-1042.

Engebrecht et al. (1987), "Nucleotide sequence of the regulatory locus controlling expression of bacterial genes for bioluminescence", Nuc. Acids Res. 15, 10455-10467.

Adhya, et al., "The Role of Gene S", (1971) *Bacteriophage Lambda*, pp. 743-746, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Baldwin et al., "The Complete Nucleotide Sequence of the lux Regulon of *Vibrio fischeri* and the luxABN Region of Photobacterium leiognathi and Mechanism of Control of Bacterial Bioluminescence" (1989) *J. Biolum. Chemilumin.*, 4:326-341.

Bedwell and Nomura, "Feedback regulation of RNA polymerase subunit synthesis after the conditional overproduction of RNA polymerase in Escherichia coli", (1986) *Mol. Gen. Genet.* 204:17-23.

Coleman et al., "Baceriophage MS2 Lysis Protein Does Not Require Coat Protein to Mediate Cell Lysis," (1983) *J. Bacteriol.*, 153:1098-1100.

Devine et al., "Nucleotide Sequence of the luxR and luixI Genes and Structure of the Primary Regulatory Region of the lux Regulon of *Vibrio Fischeri*, ATCC 7744," (1988) *Biochemistry*, 27:837-842.

Devine et al., "Identification of the operator of the lux regulon from the *Vibrio fischeri* strain ATCC7744," (1989) *Proc. Natl. Acad. Sci. USA*, 86:5688-5692.

Dunlap and Greenberg, "Control of *Vibrio fischeri* Luminescence Gene Expression in *Escherichia coli* by Cyclic AMP and Cyclic AMP Receptor Protein," (1985) *J. Bacteriol.* 164:45-50.

Dunlap and Greenberg, "Control of *Vibrio fischeri* lux Gene Transcription by Cyclic AMP Receptor Protein--LuxR Protein Regulatory Circuit" (1988) *J. Bacteriol.*, 170:4040-4046.

(List continued on next page.)

Primary Examiner—Richard A. Schwartz
Assistant Examiner—Philip W. Carter
Attorney, Agent, or Firm—Arnold, White & Durkee

[57] **ABSTRACT**

The invention relates to an expression vector system based on the regulation of bacterial luminescence (the lux gene system). The invention further relates to the construction of a precisely regulatable expression vector system which comprises a complete luxR gene in combination with an inactivated luxI gene. If the system is turned off, no significant transcription occurs of any cloned gene product when used in combination with the regulatory scheme of the invention as is demonstrated by using the bacteriophage λ lysis genes. The induction of transcription relies on the addition of exogenous autoinducer which is both inexpensive and easy-to-use and which is required in only minute amounts.

15 Claims, 2 Drawing Sheets

OTHER PUBLICATIONS

Eberhard, "Inhibition and Activation of Bacterial Luciferase Synthesis," (1972) *J. Baceriol.* 109:1101–1105.

Eberhard, "Structural Identification of Autoinducer of Photobacterium *fischeri* Luciferase," (1981) *Biochemistry,* 20:2444–2449.

Eberhard et al., "Analogs of the autoinducer of bioluminescence in *Vibrio fischeri,*" (1986) *Arch. Microbiol.,* 146:35–40.

Engebrecht, et al., "Bacterial Bioluminescence: Isolation and Genetic Analysis of Functions from *Vibrio fischeri*", (1983) *Cell* 32:773–781.

Engebrecht and Silverman, "Identification of genes and gene products necessary for bacterial bioluminescence," (1984) *Proc. Natl. Acad. Sci. USA* 81:4154–4158.

Engebrecht and Silverman, "Regulation of Expression of Bacterial Genes for Bioluminescence," In J. K. Setlow and A. Hollaender (eds.), (1986) *Genetic Engineering,* 8:31–44.

Goldberg and Howe, "New Mutations in the S Cistron of bacteriophage Lambda Affecting Host Cell Lysis," (1969) *Virology* 38:200–202.

Hastings and Weber, "Total Quantum Flux of Isotropic Sources," (1963) *J. Opt. Soc. Am.,* 53:1410–1415.

Henikoff, et al., "Finding Protein Similarities with Nucleotides Sequence Databases" (1990) *Methods Enzymol.* 183:111–132.

Humphreys, et al., "Mutagenesis of Plasmid DNA with Hydroxylamine: Isolation of Mutants of Multi-copy Plasmids," (1976) *Mol. Gen. Genet.* 145:101–108.

Joachimiak, et al. "Purification and Characterization of trp aporepressor," (1983) *Proc. Natl. Acad. Sci. USA* 80:668–672.

Kaplan, et al. "Synthesis of N-[3-OXO-(4,5-$^{3}H_{2}$)-HEXANOYL] homoserine lacone: Biologically Active Tritium-Labelled *Vibrio Fischeri* Autoinducer," (1985) *J. Labelled Compd. Radiopharm.* 22:387–395.

Kaplan and Greenberg, "Diffusion of Autoinducer Is Involved in Regulation of the *Vibrio fischeri* Luminescence System," (1985) *J. Bacteriol.,* 163:1210–1214.

Kaplan and Greenberg, "Overproduction and purification of the luxR gene product: Transcriptional activator of the *Vibrio fischeri* luminescence system," (1987) *Proc. Natl. Acad. Sci. USA* 84:6639–6643.

Konisky, "Colicins and Other Bacteriocins with Established Modes of Action," (1982) *Ann. Rev. Microbiol.,* 36:125–144.

Lanzer and Bujard, "Promoters largely determine the efficiency of repressor action," (1988) *Proc. Natl. Acad. Sci. USA* 85:8973–8977.

Meighen, "Enzymes and Genes From The lux Operons of Bioluminescent Bacteria," (1988) *Ann. Rev. Microbiol.* 42:151–176.

Michaelis and Beckwith, "Mechanism of Incorporation of Cell Envelope Proteins in *Escherichia coli,*" (1982) *Ann Rev. Microbiol.* 36:435.

Rosenberg et al. "Gene Amplification & Analysis," (1981) In, *J. G. Chirikjian* (ed.) pp. 132–164.

Shadel et al., "Use of Regulated Cell Lysis in a Lethal Genetic Selection in *Escherichia coli:* Identification of the Autoinducer-Binding Region of the LuxR Protein from *Vibrio fischeri* " ATCC 7744, (1990) *J. Bacteriol.* 172:3980–3987.

Slock et al., "Critical Regions of the *Vibrio fischeri* LuxR Protein Defined by Mutational Analysis," (1990) *J. Bacteriol.* 172:3974–3979.

Tabor and Richardson, "DNA sequence analysis with a modified bacteriophage T7 DNA polymerase," (1989) *Proc. Natl. Acad. Sci. USA* 84:4767–4771.

Ulitzur, "The Regulatory Control of the Bacterial Luminescence System—A New View," (1989) *J. Biolumin. Chemilumin.* 4:317–325.

Ulitzer and Kuhn, "The Transcription of Bacterial Luminescence is Regulated by Sigma 32," (1988) *J. Biolumin. Chemilumin.* 2:81–93.

Kemper and Hanson, "Aspects of Light Production by Photobacterium *fischeri,*" (1968) *J. Bacteriol.* 95:975–979.

Nealson et al., "Catabolite Repression of Bacterial Bioluminescence: Functional Implications," (1972) *Proc. Nat. Acad. Sci. USA* 69:1073–1076.

Nealson et al., "Cellular Control of the Synthesis and Activity of the Bacterial Luminescent System," (1970) *J. Bacteriol.* 104:313–322.

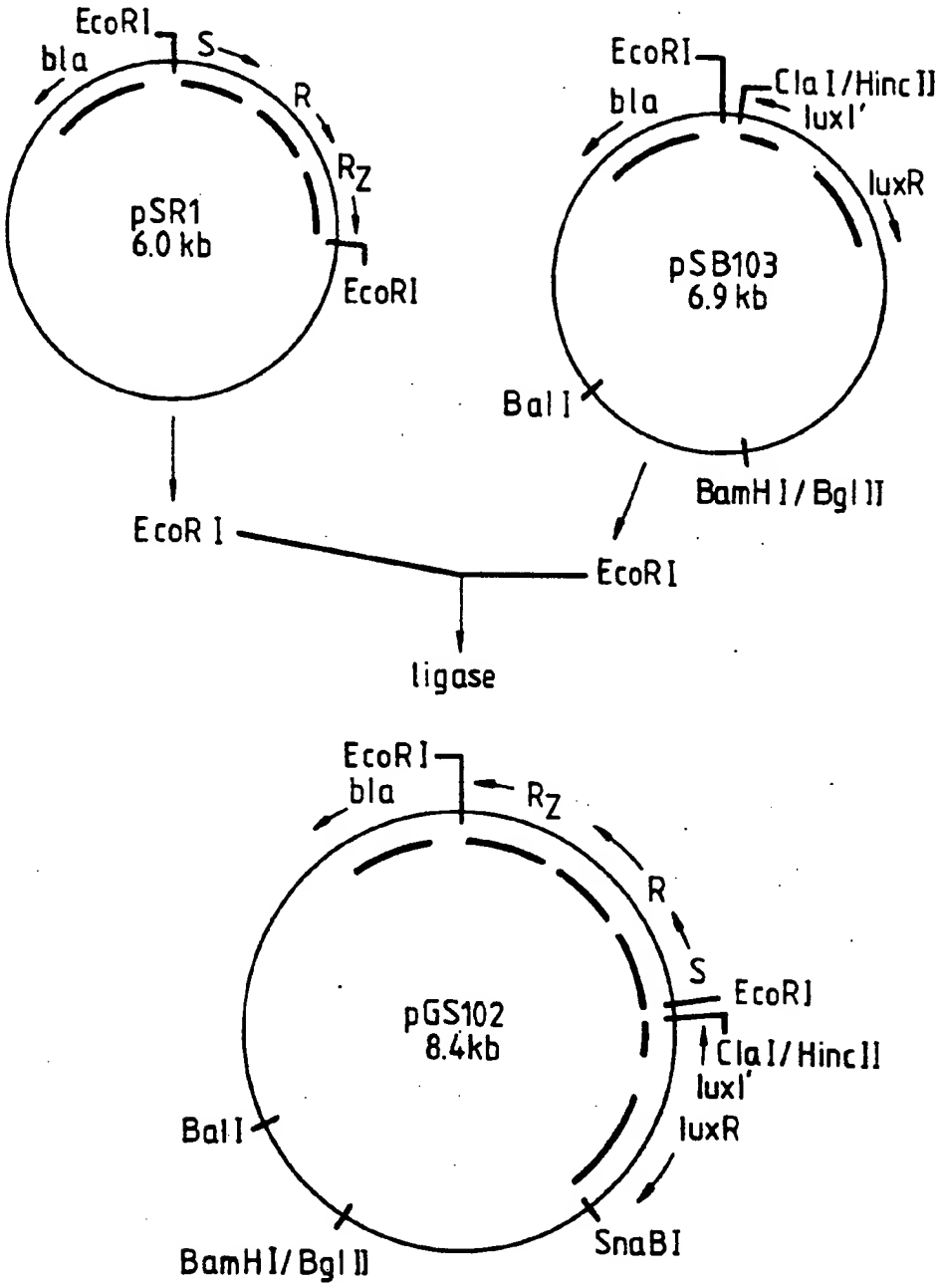

EcoRI
bla
S
R
Rz
pSR1
6.0 kb
EcoRI
EcoRI
bla
ClaI/HincII
luxI'
luxR
pSB103
6.9 kb
BalI
BamHI/BglII
EcoRI
EcoRI
ligase
EcoRI
bla
Rz
R
S
EcoRI
pGS102
8.4kb
ClaI/HincII
luxI'
luxR
BalI
SnaBI
BamHI/BglII

FIG.1

FIG.2

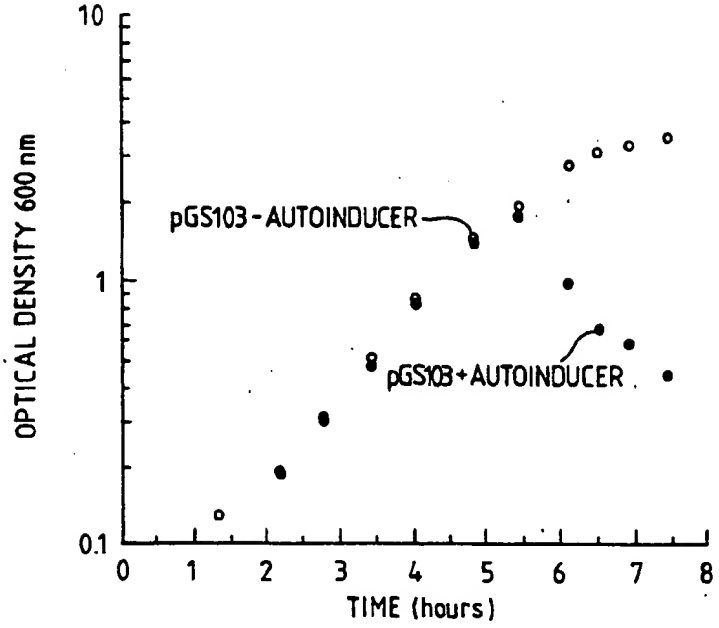


FIG.3

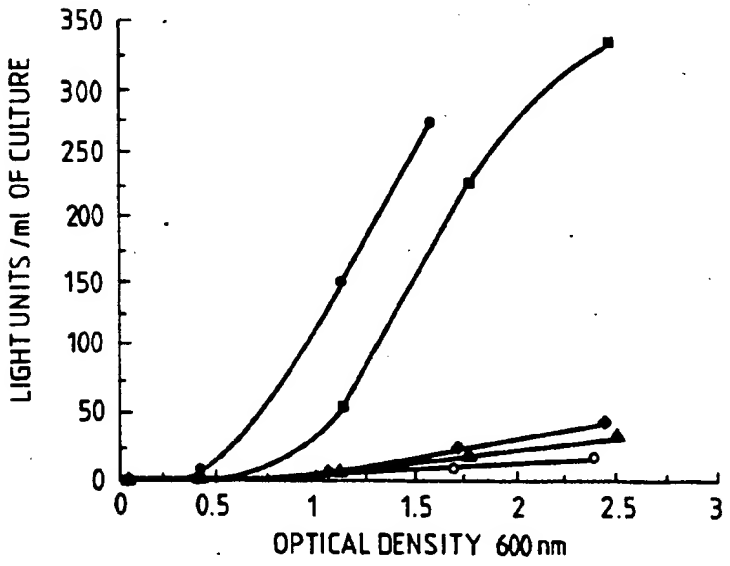
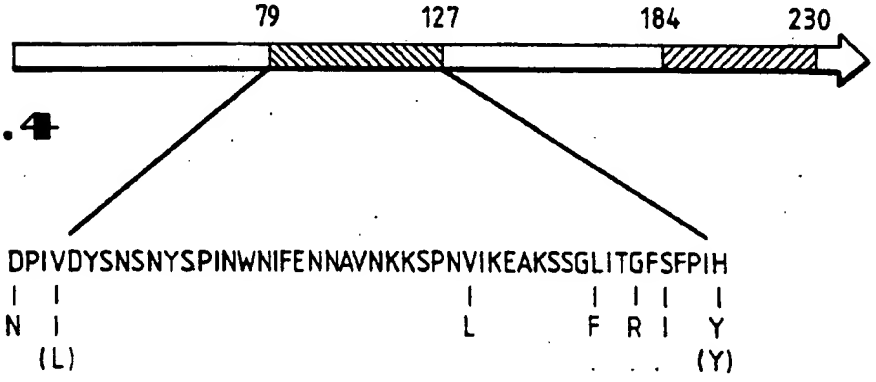


FIG.4



PRECISELY REGULATED EXPRESSION OF DELETERIOUS GENES

BACKGROUND OF THE INVENTION

The National Science Foundation provided funding used in part for this for this invention under grant RF 5311. Accordingly, the Federal Government may have certain rights in this invention pursuant to 35 U.S.C § 202.

1. Field of the Invention

The invention relates to an expression vector system based on the regulation of bacterial luminescence (the positive feedback lux regulatory circuit). The invention further relates to the construction of a precisely regulatable expression vector system which comprises a complete luxR gene in combination with an inactivated luxI gene, both of which are under the control of a common control region. The invention allows the precise temporal expression of gene products otherwise deleterious or lethal to the cell when controlled by standard expression systems. The invention further relates to the control of the expression system of the invention by an inexpensive inducer.

2. Description of the Related Art

Numerous expression systems exist for expression of gene products in bacteria. However, cloning and expression of genes which have deleterious effects on or which kill the cells in which they are expressed represents a continuing problem. Among these problem genes are a wide array of genes whose effects on the cell range from mildly deleterious to those gene products which are lethal to the cell in even minute quantities. As used herein, a deleterious gene is any gene whose expression in host cells in culture would prevent that culture from achieving the normal logarithmic growth which the culture would achieve but for the expression of the deleterious gene. Furthermore, as used herein, an expression system which is capable of stringently controlling the expression of such deleterious genes is an expression system which can sufficiently limit the expression of the deleterious gene in host cells in culture in order to allow the culture to achieve normal logarithmic growth which the culture would not achieve without the stringent control due to some level of transcription of the deleterious gene. Thus, in the case of genes whose products are lethal to the cell in even minute quantity, stringent control is that level of control which almost completely eliminates transcription of the lethal gene until released from that control.

Some deleterious genes encode gene products which if expressed in limited quantity are actually useful to the cell while if expressed in even slightly elevated quantities are deleterious to the cell. For example, such genes are epitomized by DNA-modifying enzymes such as the DNA restriction enzymes used throughout molecular biology. If allowed to be expressed in a host which is not resistant to the restriction enzyme, the host cell's own DNA is susceptible to degradation by the cloned gene's product (Rosenberg et al. 1981).

Even where a particular gene product merely stresses the host cell by its presence or by its overabundance, the production of these proteins in the cell may not be feasible. Such an effect has been observed, for instance, with overproduction of several of the subunits of *E. coli* RNA polymerase. Although it has been possible to overproduce the RNA polymerase subunits in host cells, their overexpression causes a reduction in growth rate of cultures of these cells (Bedwell and Nomura, 1986). These mildly deleterious effects represent enough of a stress to the population of cells that cells within that population, which contain a mutated version of the desired protein whose production has fewer or no deleterious effects compared to the non-mutated protein, may overgrow those cells containing the wild type protein.

Even more deleterious gene products include many proteins which become associated with the membrane of the host cells, some of which effect the cell to such a degree that the host cells are killed (Michaelis and Beckwith 1982). Some genes, in fact, code for proteins which, if expressed to any degree, even at levels as low as a few molecules of the protein per cell, lead quickly to the death of the host cell. These type of genes are typified by the class of genes encoding the lysis proteins of viruses (e.g., λ lysis protein or MS2 lysis protein) and the lytic proteins of bacteria (e.g., colicins of certain enteric bacteria) (Coleman et al. 1983; Adhya et al. 1971; Konisky 1982).

A number of patented expression systems for use in bacterial hosts have been described. In some cases, the expression systems relate to generalized expression systems. In others, specific positive regulation systems have been described. Other patented expression systems have been designed to allow relatively tight regulation. In some instances, these expression systems were individually tailored for expression of a particular protein which presented some difficulty using standard expression systems.

For instance, U.S. Pat. No. 4,782,022 appears to relate to the construction of a vector comprising a promoter of a constitutively expressed gene coupled to a gene which codes for a product capable of activating other genes required for nitrogen fixation. U.S. Pat. No. 4,775,630 appears to relate to a variant of an adenovirus control region, the regulator of which is especially sensitive to repression by products of the gene under its control. U.S. Pat. No. 4,767,708 appears to relate to the construction of a recombinant vector containing a cloned bacterial DNA polymerase I under operable control of a conditionally controllable foreign promoter. This patent notes that the foreign promoter may be a positively regulated promoter. The invention appears to be designed to overproduce DNA polymerase. U.S. Pat. No. 4,677,064 appears to relate to the use of the promoters of bacteriophage λ, P_L and N_{RBS}, in order to construct a vector capable of overexpressing human tumor necrosis factor. U.S. Pat. No. 4,634,678 appears to relate to construction of a variety of expression vectors all of which are based upon negative control systems. The patent's specification does, however, suggest the replacement of negative control sequences with positive control sequences. U.S. Pat. No. 4,578,355 appears to relate to the use of the P_L promoter of bacteriophage λ to construct a high level expression vector. U.S. Pat. No. 4,503,142 appears to relate to the construction of a class of cloning and expression vectors capable of heterologous gene expression. These vectors are based on the use of the lac promoter/operator of *Escherichia coli* (*E. coli*).

All of these systems suffer, to greater or lesser degrees, from the inability to control expression to the extent required when the gene product will kill or otherwise seriously damage the host cell if expressed. The analogy can be drawn to an electrical switch connected

functionally to a device capable of inflicting great harm to those which encounter it, even if the amount of electricity reaching the device is minimal. The design electrical engineer would find it most unsatisfactory if the only switches available were those which constantly fed the lethal device small amounts of power. Moreover, even where the prior art expression systems have provided a means for limited expression of certain deleterious genes, the likelihood that the gene will mutate in order to prevent the deleterious effects on the host cell from being realized has always caused concern. This is especially true where large scale operations have been envisioned.

Additionally, many of the prior art expression systems must rely for induction of expression either on the host's biochemical responses or on costly or awkward induction means. Moreover, many prior art expression systems suffer from the fact that the inducer is a compound routinely found in nature such as naturally occurring sugar compounds. Thus, great care must be taken to prevent inadvertent exposure of cells to extraneous sources of such commonly encountered inducers.

The present inventors are involved in research into regulation of bioluminescence in the marine bacterium *Vibrio fischeri,* which regulation has been studied extensively through cloning and genetic manipulation of the lux system in *E. coli* (Devine et al. 1989; Dunlap and Greenberg 1985; Dunlap and Greenberg 1988; Engebrecht et al. 1983; Engebrecht and Silverman 1984; Engebrecht and Silverman 1986). Expression of the lux genes in *V. fischeri* is controlled by a unique form of positive feedback regulation called autoinduction, and this pattern of regulation may be duplicated by the cloned system in *E. coli* (Engebrecht et al. 1983; Engebrecht and Silverman 1986). The autoinduction response is mediated by the production and accumulation of a small molecule, the autoinducer, which is synthesized in the presence of the luxI gene product. This product molecule presumably interacts with the luxR gene product to induce the synthesis of the enzymes required for light production. Kaplan and Greenberg (1987) were able to overproduce the luxR gene product in *E. coli,* develop a procedure for purifying this overproduced protein, but were unable to demonstrate convincingly that LuxR protein had DNA-binding activity.

The autoinducer of *V. fischeri* has been identified as N-(3-oxo-hexanoyl) homoserine lactone (Eberhard et al. 1981) and has been shown to be both freely diffusible across the cytoplasmic membrane and species specific in its ability to stimulate bioluminescence (Eberhard 1972; Kaplan and Greenberg 1985). This molecule has been synthesized in vitro and shown to function in a biological assay (Eberhard et al. 1981; Kaplan et al. 1985).

The lux genes are organized into two divergently transcribed operons, termed rightward and leftward, which are separated by a common regulatory region (Devine et al. 1988; Engrebrecht et al. 1983; Engebrecht and Silverman 1987). The luxR gene is the only known gene in the leftward operon (operon$_L$) and encodes a positive regulatory protein which, in the presence of autoinducer, stimulates transcription of the rightward operon (operon$_R$). This interaction has recently been shown to require the 20-base-pair lux operator located in the control region (Devine et al. 1989). Operon$_R$ consists of at least six genes (luxICDABE). The luxI gene encodes a protein required for autoinducer synthesis (Engebrecht and Silverman 1984), the luxC, luxD, and luxE genes encode enzymes which provide luciferase with an aldehyde substrate (Meighen 1988), and the luxA and luxB genes encode the α and β subunits of the luciferase enzyme. The sequence of the entire lux regulon from *V. fisheri* has been determined (Baldwin et al. 1989).

The current model describing the autoinduction process suggests that a low basal level of transcription of operon$_R$ leads to low-level synthesis of autoinducer by luxI. High cell density is required for autoinducer to accumulate, since it is freely diffusible across the cytoplasmic membrane. It is by virtue of the diffusible nature of autoinducer that the expression of luminescence is, in nature, cell density-dependent. If the LuxR protein, whose synthesis is regulated at the transcriptional level by the cyclic AMP-catabolite gene activator protein (cAMP-CAP) system (Dunlap and Greenberg 1985; Dunlap and Greenberg 1988), has also accumulated, it can form a complex with autoinducer capable of binding to the lux operator and stimulating transcription of operon$_R$. Positive feedback results from the presence of luxI in operon$_R$, since stimulation of rightward transcription of luxR and autoinducer leads to the production of more autoinducer by increased levels of LuxI protein. In addition to this primary regulatory circuit, several global regulatory systems in *E. coli* have been shown to interact with the lux system to affect the timing of induction of bioluminescence including the heat shock (σ^{32}) system and the SOS response (Ulitzur 1989; Ulitzer and Kuhn 1988). Thus, the positive feedback mechanism of the lux regulatory circuitry leads to the sharp induction of the enzymes required for light production.

Expression systems are needed which do not rely for their induction on expensive or otherwise inadequate induction mechanisms. This is especially important for commercial operation of bacterial fermentations of useful gene products. More importantly, however, expression systems are needed which are capable of very stringently regulating the expression of deleterious or lethal genes until such time as induction of expression can be used to express commercial quantities of their otherwise harmful gene products. If such systems were available, the expression and genetic manipulation of a wide array of otherwise lethal or deleterious gene products would be possible via the powerful capabilities of batch fermentation.

SUMMARY OF THE INVENTION

The present invention relates to an expression system capable of stringently regulating the expression of deleterious or lethal genes until such time as induction of expression can be used to express high levels of the harmful gene products. The present invention further relates to expression systems which need not rely for their induction on inadequate induction mechanisms which are typically bulky, expensive or both. The present invention, therefore, further relates to systems for the expression of a wide array of otherwise lethal or deleterious gene products using bacterial fermentation.

More specifically, the present invention relates to the construction of vectors which retain an intact luxR gene and regulatory region but which lack intact copies of all of the genes in operon$_R$, retaining only a truncated luxI gene. This arrangement affords a very stringently regulated system in which operon$_R$ transcription is controlled by the addition of an inexpensive, synthetic inducer (autoinducer), but which system now lacks the positive-feedback mechanism. A potentially lethal tran-

scriptional fusion of the lysis genes (S,R,R_z) from bacteriophage λ was created in operon$_R$ by insertion downstream of the truncated luxI gene in order to test the ability of this system to express a very lethal gene.

Such an expression system possesses two key attributes which distinguish the invention over the prior art. First, the transcription system of the invention is not as leaky as are those of the prior art. If the system is turned off, no significant transcription occurs of any cloned gene product when used in combination with the regulatory scheme of the invention. The surprising and unexpected level to which regulation can be controlled with this system was demonstrated convincingly by using the bacteriophage λ lysis genes. These bacteriophage lysis gene products are lethal to bacteria where even low transcription levels are allowed. Only a system which almost thoroughly stops transcription can be used to express such lethal proteins.

Coupled with the novel expression system, the second distinguishing attribute of the invention relates to the nature of the event which turns on transcription. The present inventors and others have found that other expression systems use either awkward or expensive events to stimulate synthesis of the cloned gene product. The stimulatory event of the present invention, however, relies on the addition of exogenous autoinducer which is both inexpensive and easy-to-use and which is required in only minute amounts. Additionally, this compound is not found routinely in nature avoiding problems of inadvertent induction found in prior art systems.

The present inventors set out to develop a flexible prokaryotic expression system utilizing the regulatory genes isolated from the marine bacterium *Vibrio fischeri*. The present inventors knew that *V. fischeri* displayed both a dramatic increase in the rate of luciferase synthesis following induction, apparently due to a unique positive feedback mechanism, and an enormous difference (ca. 10^5) in levels of luminescence in cells before and after induction. The present inventors have demonstrated that a 20 base pair inverted repeat, ACCTGTAGGATCGTACAGGT, is the binding site for the LuxR-autoinducer complex. They have also found that deletion of sequences upstream of the palindrome leads to increased transcription from the rightward promoter, indicative of a cis-acting element(s) that represses transcription in the absence of LuxR:autoinducer. Modifications of the palindrome that eliminate stimulation by LuxR:autoinducer of transcription from P_R have no effect on repression by the cis-acting mechanism(s), suggesting that the palindrome is not necessary for repression of the rightward operon. Thus, it appears that the large increase in transcription upon induction of the lux regulon is the result of at least two independent mechanisms, one positive and the other negative. These unexpected findings lead to the realization that a unique system of expression might be possible which would allow a very stringent control of genes functionally connected to such an expression system.

The objective of the present inventors has been, therefore, to design and construct a unique vector in which induction is controlled by the addition of autoinducer. The requirement for autoinducer is accomplished by deletion of the luxI gene so that expression of the cloned gene can only be accomplished by addition of autoinducer. The basal level of rightward transcription is very low because the lux system is tightly repressed in the absence of autoinducer. This is an important consideration if the protein of interest is toxic to the host. Expression can be postponed until late in growth without adversely affecting the bacterium. This point was made very clear by the unexpectedly successful cloning and expression of the lysis genes from bacteriophage λ under control of operon$_R$ which allows control of cell lysis by autoinducer addition. In the absence of autoinducer, these lethal genes can be propagated without adversely affecting the host cells.

Accordingly, an expression system has been invented which consists of a lux regulatory circuit connected to and capable of stringently regulating expression of a gene. In a preferred embodiment of the present invention, at least one product of the cloned gene will be deleterious to the host cells used to express the gene. The deleterious effect may be one which directly occurs as a result of the presence of the gene product in the cell such as with a lysis protein. However, the deleterious effect may equally well be an indirect effect such as where membrane jamming occurs which eventually leads to the death of a cell.

The expression system may be used independent of other regulatory systems. Alternatively, one embodiment of the invention would include the use of the lux expression system operably linked to a second regulatory system or systems in order to achieve control of the second regulatory system by the lux expression system.

In this manner, the binding of the LuxR-autoinducer complex can also be adapted to perform a negative regulatory role. If the recognition sequence for LuxR-autoinducer is situated near a promoter such that its binding prevents the binding of RNA polymerase, then it becomes a repressor. Over two thousand-fold induction has been achieved with this strategy using lac repressor and its operator (Lanzer and Bujard 1988).

The lac system is controlled by inducers, usually isopropylthio-β-galactoside (IPTG) or allolactose, which, when bound to the lac repressor, reduce its affinity for the lac operator, thereby relieving repression. However, if LuxR were used as a repressor, its effector (autoinducer) would act as a co-repressor, much like L-tryptophan does with the trp repressor. The trp repressor-operator system can be "induced" if 3-β-indoleacrylic acid (IAA), a tryptophan analogue, is administered while sufficient L-tryptophan is present in the cell to cause repression (Joachimiak et al. 1983). The IAA competes with L-tryptophan for the trp repressor but the repressor-IAA complex does not bind tightly to the trp operator, so repression is relieved.

Eberhard and co-workers have synthesized autoinducer analogues and have shown several of these to be potent competitive inhibitors of autoinducer in *Vibrio fischeri* (Eberhard et al. 1986). It is possible that one or more of the analogues, like IAA with the trp repressor, could relieve repression by LuxR. If this approach proves impractical, relief of repression can be accomplished by harvesting the cells and resuspending them in fresh, autoinducer-free, culture medium.

The advantage of the LuxR "repressor" configuration is that one can make use of the specificity of LuxR for its economical, non-metabolizable effectors without relying on the specific protein-protein contacts between LuxR and prokaryotic RNA polymerases that are needed for transcriptional activation. Because only DNA binding is required of LuxR for repression, and because of the apparent permeability of membranes to autoinducer, this technology and its benefits could be

extended to distantly related prokaryotic systems (e.g. gram-positive bacteria) and perhaps even to eukaryotic systems like yeast and cultured animal or plant cells.

In a highly preferred embodiment of the present invention, the lux regulatory circuit consists of a luxR gene derived from the operon$_L$ of a lux regulatory circuit. The luxR gene is under regulation of the control region of the lux regulatory circuit and operably linked to it. The lux control region is further connected to a portion of operon$_R$. The rightward operon normally consists of intact and sequentially oriented genes, luxICDABE, but in this highly preferred embodiment the operon only retains a truncated luxI gene in order to disrupt the normal positive feedback associated with the rightward operon. The lux regulatory region of the present invention may be obtained from a number of different bioluminescent bacteria as will be well understood by those of skill in the art. In certain preferred embodiments, the lux regulatory circuit is isolated from *Vibrio fischeri.*

The expression system of the present invention is designed to allow expression of any deleterious gene products. However, in certain preferred embodiments the expression system may be used to express the product of a gene which, when expressed, is lethal to the host cell. Expression of a particularly lethal gene is provided in a preferred embodiment where lysis genes S, R and R_z of bacteriophage λ are expressed using the expression system of the present invention. Similar genes would include the lysis genes of the MS2 virus and the lytic peptides of the enteric bacteria such as colicins.

Additionally, the expression system of the present invention is designed such that induction of expression is controlled by addition of an inducer to the environment of the host cells containing the expression system. Naturally occurring inducer may be used. In one embodiment, naturally occurring autoinducer produced endogenously within the host cell carrying the expression system of the present invention may be achieved as a function of the density of the host cells in culture. Since luxI, whose product is necessary for the synthesis of endogenously-produced autoinducer, is the first gene in operon$_R$, LuxR stimulation leads to the synthesis of more autoinducer and a positive feedback loop is created leading to a sharp induction of operon$_R$. The timing of induction is therefore a function of the basal level of rightward transcription, which determines the rate of initial accumulation of autoinducer. Vectors containing the intact luxR and luxI genes will allow the density-dependent regulation to be imposed on a target gene cloned downstream of luxI in operon$_R$. The timing of induction can then be altered through mutagenesis of the rightward promoter, allowing variable basal levels of transcription to be achieved and induction of the target gene to occur at a chosen cell density.

However, in a preferred embodiment, the inducer will be an exogenously added inducer. In general and overall embodiments, the inducer must be able to induce the expression of the leftward operon to a degree adequate to initiate transcription in the rightward operon (See, e.g., Eberhard et al. 1986 for functional analogs). In a most preferred embodiment, the inducer will be the inexpensive and readily-synthesized molecule, N-(3-oxo-hexanoyl) homoserine lactone. In certain other embodiments, the inducer will be an autoinducer characterized as a small diffusible molecule capable of the requisite capability to control expression in combination with the regulatory circuits of the present invention.

It is, of course, possible to use the expression system of the present invention in the native Vibrio host or in related bacterial species. However, in a preferred embodiment, the expression system will be used in conjunction with *Escherichia coli* cells.

The present invention also relates to construction of vectors designed to carry lux regulatory circuitry associated with the expression systems of the invention. In a preferred construction, such vectors will contain a truncated luxI gene functionally connected to a multiple cloning site downstream of the truncated portion. Such multiple cloning sites are well known in the art and consist of a number of restriction endonuclease sites for ease of cloning gene-containing fragments downstream of the luxI truncation.

A method for expression of a deleterious gene is provided by the present invention as well. The steps include constructing a vector with a lux regulatory circuit, functionally connecting a deleterious gene to the lux regulatory circuit, transforming the vector containing the regulatory circuit and deleterious gene into a host cell and growing the resulting host cells to a late phase of growth in which there are many such cells with many such vectors and then inducing expression of the deleterious gene by adding an exogenous inducer to the growth medium of the bacterial culture.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Construction of the lysis vector pGS102. Small arrows indicate the 5'-to-3' direction of the gene indicated, and large arrows indicate cloning steps taken during the construction of the pGS102 plasmid (kb=Kilobases). Deletion of the DNA segment from SnaBI to BalI shown in pGS102 results in the preferred construction pGS103.

FIG. 2. Growth of cultures of *E. coli* TB1 carrying the plasmid pGS103 and demonstration of autoinducer-dependent cell lysis. Autoinducer (5 μM) was added from the beginning of the experiment where indicated.

FIG. 3. Autoinducer response of pJHD500 and three LuxR− pJHD500 derivatives, Symbols: ● and o, pJHD500-wild type LuxR; ■, H127Y; ◆ V82L; ▲, V821. Open symbols indicate no autoinducer added to the culture, and closed symbols indicate the addition of 2.5 μM autoinducer from the beginning of the experiment.

FIG. 4. Critical regions of the LuxR protein defined by random mutagenesis and the primary sequence of the autoinducer-binding region (▮). Boldface type indicates random mutations isolated in this study which map between residues 79 and 127. Open type indicates random mutations isolated by Slock et al. (1990) which map between residues 79 and 127. Letters in parentheses indicate amino acid changes introduced by site-directed mutagenesis. The second critical region is defined by mutations isolated by Slock, et al. (1990) and the H217Y mutation isolated in this study (※).

DESCRIPTION OF PREFERRED EMBODIMENTS

The following examples describe, in detail, the steps required to practice the present invention and demonstrate the utility of the expression system of the present invention when used in combination with a very lethal gene product, the lysis genes of λ bacteriophage. In particular, the construction of a plasmid vector which retains an intact luxR gene and regulatory region but

lacks all of the genes in the operon$_R$, retaining only a truncated luxI gene is described. This arrangement resulted in an expression system in which operon$_R$ transcription is controlled by the addition of synthetic autoinducer but which lacks the positive-feedback mechanism. The potentially lethal transcriptional fusion of the lysis genes (S,R,R$_z$) from bacteriophage λ was created in operon$_R$ by insertion downstream of the truncated luxI gene demonstrating the utility of the invention for the expression of a very lethal lytic gene product. In another demonstration of the utility of the present invention, vector (pGS102) was used in a lethal genetic selection in conjunction with a subsequent luminescence screen to recover point mutations in the luxR gene.

EXAMPLE I

Enzymes and chemicals. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc., or Boehringer Mannheim Biochemicals. Klenow fragment of *E. coli* DNA polymerase I and modified T7 DNA polymerase (Sequenase) were purchased from United States Biochemicals. ATP and deoxyribonucleotides were obtained from Pharmacia LKB. Radiolabeled dATP was obtained from Dupont, NEN Research Products. Hydroxylamine hydrochloride and n-decanal were obtained from Sigma Chemical Co. All other chemicals were of the highest quality commercially available.

Cell strains. All growth experiments were performed using *E. coli* TB1 [hsdR Δ(lac pro)]. *E. coli* CA8404 (crp* was a gift of Pete Greenberg, but is readily obtainable by methods known well to those of skill in the art) was used to achieve better complementation with luxR in trans. This strain produces a mutant CAP which does not require cAMP to activate transcription from cAMP-CAP-regulated promoters.

Plasmid constructions. Construction of the lysis vector pGS102 is summarized in FIG. 1. pGS102 was constructed from the plasmid pSB101, which contains a BglII restriction fragment harboring the bioluminescence genes luxR, -I, -C, and -D' cloned into the BamHI site of pBR322 with the transcription of operon$_R$ oriented opposite that of the tet gene. To eliminate production of the autoinducer via the luxI gene product, pSB101 was digested with ClaI, filled with Klenow fragment, partially digested with HincII, and ligated to yield plasmid pSB103 containing a truncated luxI gene fused at the ClaI site of pSB101. The lysis genes of bacteriophage λ (S, R, and R$_z$) were isolated on a 1.5-kilobase EcoRI restriction fragment from plasmid pSRI, which is a derivative of plasmid pRGI (Raab et al. 1986) with the unique HindIII site converted to an EcoRI site by using synthetic adapters. This EcoRI fragment was subsequently ligated into the EcoRI site of pSB103 to yield pGS102. Restriction analysis was used to screen for the proper orientation of the lysis genes in the pGS102 construction which generated a transcriptional fusion between operon$_R$ of the *V. fischeri* lux genes and the λ lysis genes, which were now located downstream of the truncated luxI gene.

A SnaBI-BalI deletion of pGS102 was constructed to remove *V. fischeri* DNA that was downstream of the luxR gene in the operon$_L$. This construction was called pGS103 and exhibited the same lysis phenotype as pGS102.

The construction of plasmid pJHD500 has been described earlier (Devine et al. 1989). This plasmid is similar to pGS102, except the lux A and luxB genes, encoding the subunits for the luciferase enzyme from *Vibrio harveyi*, were cloned downstream of the truncated luxI gene, creating a bioluminescent transcriptional reporter for operon$_R$. DNA containing mutations in the luxR gene generated in pGS102 and pGS103 were subcloned into pJHD500 in order to quantitate the ability of the mutant LuxR proteins to stimulate rightward transcription.

Growth of cultures and measurement of the Extent of Cell Lysis. The ability of the mutant LuxR proteins to respond to autoinducer was determined by monitoring cell growth and aldehyde-stimulatable luminescence of samples withdrawn from cultures grown in 50-ml culture flasks containing (initially) 12 ml of Luria-Bertani (LB) medium with carbenicillin (100 μg/ml) at 30° C. in a New Brunswick model G76 gyratory water bath shaker at 200 rpm. Inoculation was from overnight cultures grown at 30° C. and diluted 1/200 (vol/vol). Samples (1 ml) were removed at various times during growth. Cell density was determined as the optical density at 600 nm (OD$_{600}$) by using a Milton Roy Spectronic 601 spectrophotometer. The same samples were then used for luminescence measurements in vivo by transferring the sample into a 20-ml scintillation vial, placing the vial in a photometer, and injecting 1 ml of a sonicated solution of n-decanal (10 μl of aldehyde per 10 ml of LB medium). Peak light emission was monitored by using a photomultiplier-photometer for which 1 light unit represents 9.8×10^9 quanta/s, based on the liquid light standard of Hastings and Weber (1963).

Data depicting cell lysis were collected by monitoring culture growth in 125-ml culture flasks containing 20 ml of LB medium with carbenicillin (100 μg/ml) at 30° C. in a New Brunswick Aquatherm water bath with shaking at 100 rpm. Duplicate cultures were inoculated by dilution (1/200 [vol/vol]) of an overnight culture grown at 30° C. with an additional of autoinducer of one of the cultures immediately after inoculation. Cell lysis was detected as a drop in the OD$_{600}$ of the culture.

Pure autoinducer was synthesized by the method of Eberhard et al. (Eberhard et al. 1981) and was stored as a 50 mM solution in water at 4° C. This stock was further diluted into the growth medium to the desired concentration. The concentration of autoinducer was calculated by using the weight of the dried, purified material and a molecular mass of 213 g/mol for autoinducer.

Colonies containing pJHD500 and its luxR mutant derivatives were screened for luminescence on solid medium by applying n-decanal to the lid of a petri dish and observing the glowing colonies in a dark room.

It is important to note that certain problems were encountered in this study while adapting the lysis method to the lux system which were mainly a result of different temperature requirements for the lux system isolated from a marine bacterium, and the lysis genes which normally function in the enteric bacterium *E. coli*. For example, when *E. coli* carrying a lysis plasmid was grown at 37° C., cell lysis was observed in the absence of autoinducer. However, cell lysis was not observed in the absence of autoinducer when the cultures were grown at 30° C. or lower. This apparently resulted from either an increased basal level of transcription of operon$_R$ at 37° C. or the ability of the protein products of lysis genes to function more efficiently at the higher temperatures. Temperature therefore appears to be one parameter which can be adjusted to

optimize the conditions for the lysis selection and may provide a means for controlling the lethality of the lysis genes, allowing selection of a variety of mutants.

Mutant selection in liquid medium. A variety of growth conditions were used for lysis selection in liquid medium. Temperatures were varied between 24° and 37° C., and M9 minimal medium supplemented with glycerol (0.2%), proline (40 μg/ml), and thiamine (0.001%) was used as well as LB medium. Autoinducer concentrations were varied between 0.5 and 5 μM. In all experiments, 5-ml cultures of *E. coli* TB1 containing plasmid pGS102, prepared by inoculation from overnight cultures by dilution (1/100) into medium containing carbenicillin (100 μg/ml), were used. Duplicate cultures were incubated with and without autoinducer until cell lysis was observed visually as a loss of the turbidity of the culture. Cultures were then diluted into fresh medium, plated onto solid LB medium, and incubated at 30° C. to allow growth of surviving cells. In some cases, the lysed cultures were pelleted, resuspended in fresh autoinducer-containing medium, and taken through a second lysis induction (double-induction experiment).

Hydroxylamine mutagenesis and screening on solid medium. Hydroxylamine mutagenesis was done essentially as described previously (Humphreys et al. 1976). Purified pGS103 (2.5 μg) was suspended in 250 μl of a 1M hydroxylamine solution at pH 6.0. The hydroxylamine solution was prepared by mixing 125 μl each of a 2M hydroxylamine solution (0.7 g of hydroxylamine hydrochloride dissolved in 0.56 ml of 4N NaOH and adjusted to 5 ml with sterile water) and a 2×TE solution (200 mM Tris Cl, pH 6.0, 2 mM EDTA). This mutagenesis mixture was incubated at 65° C. for 35 min, and the modified DNA was precipitated with 2 volumes of ethanol after the addition of ammonium acetate to 1M. The pellet was suspended in TE (pH 8.0), and 0.25 μg was used to transform competent *E. coli* TB1. Transformed cells were plated in duplicate onto solid. LB medium containing carbenicillin (100 μg/ml) with and without 5 μM autoinducer. Mutant colonies were screened as normal opaque colonies on autoinducer-containing plates against a background of translucent colonies (see, Example II, infra).

DNA sequencing. Double-stranded plasmid DNA was prepared from overnight cultures by the alkaline lysis method (Maniatis et al. 1982). The DNA pellets were then treated with RNase A and precipitated with polyethylene glycol 8000. The purified DNA was then denatured with NaOH and used as template for sequencing by the dideoxy-chain termination method using modified T7 DNA polymerase (Sequenase) (Tabor and Richardson 1987). Sequencing primers used to sequence luxR have been described elsewhere (Devine et al. 1989).

Site-directed mutagenesis. Site-directed mutagenesis was done by the method of Kunkel et al. (1987), with slight variations. Single-stranded uracil-containing DNA isolated from phagemid-infected cells was used as a template for the mutagenesis reactions. Purification of the template was done as described earlier (Devine et al. 1989), except the starting plasmid was pVFS185, which is a derivative of pTZ18R (Pharmacia) containing a SacI restriction fragment harboring most of luxR, all of luxI and luxC, and a portion of luxD.

EXAMPLE II

Demonstration that the lysis phenotype is under lux control. The lysis vectors pGS102 and pGS103 both allowed expression of the lysis phenotype to be controlled by the addition of synthetic autoinducer. A SnaBI-BalI deletion of pGS102 was done to create pGS103 (FIG. 1). The deleted sequences consisted of uncharacterized *V. fischeri* DNA and a portion of the pBR322 vector. Removal of these sequences had no effect on the induction of cell lysis by the addition of autoinducer. A Budapest Treaty Deposit was made prior to the filing of the present patent application with the United States Patent and Trademark Office to the American Type Culture Collection of pGS103 and that deposit has been assigned accession number ATCC 40830.

When autoinducer was added from the beginning of the growth experience or at an early point in growth, a lag was observed prior to cell lysis while LuxR protein accumulated. A typical lysis curve is shown in FIG. 2. At 30° C. in LB medium, with autoinducer added at the beginning of the experiment, cell lysis was observed as a decrease in the OD_{600} of the culture between 4.5 and 5 h after initial inoculation of the culture (OD_{600}, 1.2), followed by a steady decrease in OD_{600} during the next several hours. When autoinducer was added at a later point during culture growth, the lag period was decreased. The lag could be almost completely eliminated if *E. coli* CRP* was used, since the stimulation of luxR expression by cAMP-CAP does not require the accumulation of cAMP in this strain (data not shown). These observations suggest that the timing of induction was mainly a function of the cAMp-CAp stimulation of luxR expression.

Selection of mutants following lysis induction in liquid medium. Selection of lux regulatory mutants was accomplished by the addition of autoinducer to cultures grown in liquid medium at 24° C. and allowing the culture to incubate overnight (12 to 14 h) with shaking. Cells surviving the lysis induction were then grown on solid medium. Two problems were encountered with this procedure. First, a background of colonies was observed which retained the ability to lyse when screened in liquid medium for a nonlysing phenotype. Second, a considerable proportion of the colonies isolated from the selection which did retain a nonlysing phenotype carried plasmids which had suffered deletions and/or other rearrangements of the original pGS102 plasmid. The former problem was partially eliminated by pelleting the cells from the initial lysis induction, suspending them in fresh autoinducer-containing medium, and taking them through a second lysis induction under identical conditions. Dilutions plated from these cultures exhibited a lower background of surviving nonmutant colonies.

From the double-induction experiment in M9 medium at 24° C., 18 colonies, which were presumably mutant, were picked and grown overnight to isolate of plasmid DNA and to screen for a nonlysing phenotype in liquid medium. All 18 mutants exhibited a nonlysing phenotype, and 13 of these 18 isolates had wild-type restrictions patterns for the pGS102 plasmid. To screen for mutations which were not plasmid-borne, the plasmid DNA from the remaining 13 mutants was retransformed into wild-type *E. coli* and again screened for lysis in response to autoinducer in liquid medium. By this criterion, six of these mutants (L2S2, L2S9, L2S14,

L2S18, L2S20, and L2S21) were results of non-plasmid borne mutations and were probably results of *E. coli* chromosomal mutations which prevented cell lysis. The remaining seven mutants were judged to be plasmid borne and were next screened for luxR null phenotypes by checking for lysis in the presence of autoinducer with luxR supplied in trans on a compatible plasmid (pAC102). All seven of the mutants (L2S3, L2S5, L2S7, L2S8, L2S10, L2S15, and L2S17) exhibited a luxR null phenotype. The nucleotide sequences of the luxR genes from the luxR null mutants were determined, and the results are shown in Table 1. No mutation was identified within the luxR coding region for the L2S10 and L2S15 isolates.

TABLE 1

	Summary of luxR point mutations isolated by variations of the lysis selection		
Mutation designation	luxR nucleotide change[a]	LuxR amino acid change	Lysis selection[b]
L2S3	T-114 to A	C-38 to TGA (stop)	L
L2S5	G-325 to T	V109L	L
L2S7	G-368 to T	S123I	L
L2S8	G-352 to T	L118F	L
L2S17	T-502 to G	Y-169 to TAG (stop)	L
	C-508 to A	R170R (silent)	
XS-2	G-244 to A	V82I	S
XS-3	C-649 to T	H217Y	S
XS-4	G-235 to A	D79N	S

[a]Positions are numbered from 1 starting with the A of the AUG start codon for luxR indicated by Devine et al. (1988).
[b]L, Lysis selection in liquid medium; S, lysis screen on solid medium after hydroxylamine mutagenesis (see, Example 1, supra).

Hydroxylamine mutagenesis and lysis screen on solid medium. Attempts to select mutants by the lysis technique on autoinducer containing solid medium resulted, as with the liquid medium selection, in a large background of colonies which retained a lysing phenotype when screened in liquid medium. The desired mutant colonies could, however, be discriminated from the background of surviving nonmutant colonies as a normal versus translucent phenotype. The translucent phenotype was presumably due to a heterogeneous population of lysed and unlysed cells. In order to increase the frequency of mutant colonies, the plasmid pGS103 was modified by reaction with hydroxylamine in vitro prior to screening for mutants on autoinducer-containing solid medium. By this method, approximately 15% of the colonies were of the mutant phenotype in the hydroxylamine experiment, whereas in the control experiment of unmodified plasmid, mutant colonies were observed only at very low frequencies. Twenty mutant colonies were isolated with this screen, and plasmid DNA was prepared for further study.

Quantitation of autoinducer response of luxR point mutations. To recover mutations of the luxR gene, restriction fragments containing portions of the luxR gene from the 20 mutants isolated by the hydroxylamine experiment described above were subcloned into the bioluminescent rightward reporter vector pJHD500 and screened as dim colonies on autoinducer-containing solid medium. Colonies of cells containing pJHD500 with a wild-type luxR were bright under these conditions. Eight dim mutants were picked from this screen, five of which possessed a wild-type restriction pattern, indicating an intact luxR gene. Three of these were sequenced and shown to contain point mutations in luxR resulting in changes in the LuxR amino acid sequence of Val at position 82 to Ile, hereinafter designated as V821, H217Y, and D79N (Table 1). In addition, the three missense mutations and the L2S3 nonsense mutation isolated by the liquid lysis induction were subcloned into pJHD500 to allow transcription from operon$_R$ to be measured. To screen for mutations which could respond to higher concentrations of autoinducer, cells containing the luxR mutant derivatives of pJHD500 were replica plated onto solid medium with and without 5 μM autoinducer. The LuxR protein with the amino acid change of V821 was the only mutant observed by a visual screen to respond to this (elevated) concentration of autoinducer. To further examine the autoinducer response of the change at position 82, a second mutation was introduced by site-directed mutagenesis which changed the valine at this position to leucine instead of isoleucine.

A similar genetic study of the LuxR protein from *V. fischeri* MJ1 has been reported by Slock et al. (1990). These authors describe the isolation of several LuxR missense mutations, one of which was observed to respond to higher concentrations of autoinducer. This mutation resulted in the replacement of the histidine at position 127 with tyrosine. In order to quantitate the autoinducer response of this mutation with out reporter vector, the same mutation was constructed by site-directed mutagenesis and subcloned into pJHD500. The change was introduced by site-directed mutagenesis rather than by subcloning of the Slock et al. (1990) mutation because of the two different strains of *V. fischeri* which were used (ATCC 7744 and MJ1) and which have been shown to have differences in the amino acid sequence at four positions within the LuxR protein (Devine et al. 1989). Thus, the present inventors wanted to ensure that the observed phenotype was the result of the change at position 127 and not due to strain differences in the LuxR protein.

Growth curves and luciferase assays in vivo were done for the total of nine luxR mutations cloned in the reporter vector pJHD500. The autoinducer response was measured as the ability of the mutations to stimulate operon$_R$ transcription in the presence of autoinducer (2.5 μM) above the basal level observed in the absence of autoinducer. The results are summarized in Table 2, and complete growth curves of those mutations which respond to autoinducer are shown in FIG. 3. The results presented in FIG. 3 and Table 2 demonstrate that the change of V821 responded with an activity of approximately 70% that of the wild-type protein (23-versus 33-fold stimulation above the basal level at an OD_{600} of 1.5). The site-directed changes of V82L and H127Y responded to a much lower extent, giving only 8 and 6% of the stimulation of the wild-type protein, respectively. The change of D79N was observed to reproducibly give stimulation of about 1% that of the wild-type protein at a 10-fold-higher autoinducer concentration than that used in the above experiments (data not shown). Both of the position 82 mutants, as well as the H127Y mutant, were screened for response to a range of autoinducer concentrations. With all three of these variants, it was found that elevated levels of autoinducer were required to compensate for the lesion (data not shown). None of the remaining mutations exhibited significant autoinducer-dependent stimulation (Table 2).

TABLE 2

Autoinducer response of luxR point mutations measured in the transcriptional reporter vector pJHD500

LuxR variant of pJHD500	Light units/ml[a] Without autoinducer	With autoinducer (2.5 μM)	Stimulation (fold)	% Stimulation
pJHD500	8.0	265	33	100
L2S3	9.0	9.0	None	0
D79N	9.0	10.0	None	0[c]
V82I	7.5	175	23	70
V82L	8.0	20.0	2.5	8
V109L	7.0	8.0	None	0
L118F	8.0	8.0	None	0
S123I	6.5	6.0	None	0
H127Y	8.0	15.0	1.9	6
H217Yd	2.0	2.3	None	0

[a]Peak light emission measured from 1 ml of culture at an OC_{600} of 1.5.
[b]Values are given relative to the stimulation achieved by the wild-type LuxR protein encoded by the pJHD500 construction.
[c]At a 10-fold-higher autoinducer concentration, the response of D79N is about 1% that of the wild-type protein.
[d]The H217Y data were collected during a different growth experiment under a different set of conditions than was used for the other mutations shown in Table 1 (100 rpm, 29° C., 2.5 μM autoinducer).

Location of random mutations in luxR. The locations of the randomly generated mutations isolated in this study are shown in FIG. 4 along with two mutations isolated by Slock et al. (1990), G121R and H127Y, which reside in the same region of the LuxR primary sequence. A total of seven randomly generated missense mutations occur within a 49-amino-acid stretch of the LuxR protein spanning residues 79 to 127 labeled as the autoinducer-binding region in FIG. 4 (see, Example III, infra). One mutation isolated during the luminescence screen, H217Y, occurred within a second critical region in LuxR spanning residues 184 to 230 and defined primarily by mutations isolated by Slock et al. (1990) (FIG. 4).

EXAMPLE III

Several features of the lysis gene cassette from bacteriophage λ make it generally useful as the lethal component in a lethal genetic selection. The results of an exhaustive mutational analysis of the bacteriophage λ S gene revealed that host mutations which confer resistance to the lethal action of the S protein are not recovered, since the S protein apparently acts alone in forming the lethal pore in the cytoplasmic membrane (Raab et al. 1986). This is not true for many other lethal proteins, which require interaction with host components in order to exert their lethal functions. The S gene is the only lethal gene of the three comprising the lysis cassette, and it consists of only 107 codons. The small size of the S gene makes it a small target for mutation, and therefore, the frequency of recovering mutations in the lysis cassette which prevent lethality is very low. In addition, many of the codons constituting the S gene are not mutable by transitions to "knockout" missense mutations or non-sense mutations which result in loss of S protein function. Lastly, a simple secondary screen can distinguish between the desired transcriptional control mutants and unwanted S gene mutants. This screen utilizes the ability of a limited amount of chloroform to substitute for S protein pore formation by disrupting the cell membrane and allowing the diffusion of accumulated murein transglycosylase into the periplasm (Goldberg and Howe 1969). The murein transglycosylase, the product of the λ R gene, is responsible for degradation of the peptidoglycan in the *E. coli* cell wall (Bienkowska-Szewczyk et al. 1981). Transglycosylase accumulation does not require S gene function; therefore, mutations which decrease transcription of the lysis cassette will not lyse even in the presence of chloroform, while mutations in the S gene will allow lysis in the presence of chloroform since the transglycosylase can still accumulate and is free to enter the periplasm.

In this study, the λ lysis cassette was used to isolate mutations in the luxR gene from *V. fischeri* with *E. coli* as a host for the cloned lux genes. A transcriptional fusion was created between the bacteriophage λ lysis genes and $operon_R$ by insertion downstream of a truncated luxI gene. The resulting plasmid allowed cell lysis to be controlled by the addition of synthetic autoinducer to the growth medium. Mutations in the luxR gene generated by variations of this lysis selection were subcloned into the luminescent reporter vector pJHD500, which created both a secondary screen for defective LuxR proteins and a method for quantitating the ability of these LuxR variants to respond to autoinducer.

A total of seven randomly generated missense mutations have been characterized, and by DNA sequence analysis, the lesions have been shown to occur within a 49-amino-acid stretch of the LuxR primary sequence. An additional mutation introduced by site-directed mutagenesis changed the valine at position 82 to leucine. A change of valine to isoleucine at position 82 of LuxR resulted in a protein with 70% of the autoinducer-dependent transcriptional stimulation capacity of the wild-type protein, while changing this same valine to a leucine resulted in a protein exhibiting only 8% of the wild-type response (Table 2). The ability of such conservative changes at position 82 of LuxR, valine to isoleucine and valine to leucine, to dramatically affect the autoinducer response of the resulting proteins suggests that this residue may be involved in direct interaction with the autoinducer molecule. A total of four luxR mutations yielded proteins which exhibited an ability to stimulate transcription of $operon_R$, albeit to lower-than-wild-type levels, while the remaining luxR mutations did not allow any detectable autoinducer-dependent stimulation, even at elevated concentrations of autoinducer. The clustering of the seven randomly generated mutations within the region spanning residues 79 to 127 of the LuxR protein demonstrates that this region of the protein is critical for activity. The ability of several mutations within this region to respond to elevated concentrations of autoinducer suggests that the autoinducer-binding site is composed, at least in part, of amino acids residing within this region.

The possibility that the mutations in the proposed autoinducer-binding region may result in the production of unstable proteins which could give rise to the defective autoinducer response phenotype observed cannot be ruled out by the data of the present invention. However, two additional mutations isolated by Slock et al. (1990), G121R and H127Y, were shown by Western immunoblot analysis to be synthesized in vivo at levels comparable to those of the wild-type protein. These results increase the likelihood that the mutant proteins of the present invention are likewise produced at wild-type levels. This is especially true of the mutations at position 82 of luxR which both give stimulation greater than does the H127Y protein, which is synthesized at wild-type levels. It is difficult to imagine a situation in which the proteins with position 82 mutations could

give greater stimulation if they were synthesized at levels lower than wild-type levels.

The results of this mutational study of the LuxR protein, coupled with those of Slock et al. (1990), demonstrate that at least two functional regions exist in the LuxR protein. One region spans amino acids 79 to 127 and is proposed to be an autoinducer-binding region, and the other region spans amino acids 184 to 230 (FIG. 4). Although no experimental evidence demonstrates that the mutations isolated by Slock et al. (1990) and the mutation of H217Y isolated in this study, which resides in this second critical region, are in fact defective in DNA binding, there is some recent evidence which supports the hypothesis that a carboxy-terminal DNA-binding region exists in LuxR. Alignment of amino acid sequences similar to those of the LuxR protein by Henikoff et al. (1990) revealed that a carboxy-terminal region of LuxR has a sequence similar to regions within nine other diverse bacterial proteins, including five known activator proteins (FixJ from *Rhizobium meliloti*, MalT and UhpA from *E. coli*, GerE from *Bacillus subtilis*, and RcsA from Klebsiella aerogenes). The apparently homologous regions revealed have been predicted to form a helix-turn-helix DNA-binding motif at a common position which includes residues within the region defined by mutations residing between positions 184 and 230 in LuxR. Further biochemical evidence is required, however, before this region can be unequivocally defined as the DNA-binding region of the LuxR protein.

The primary regulatory circuit controlling the induction of bioluminescence in *V. fischeri* appears to require three interacting elements: the LuxR protein, the autoinducer molecule, and the lux operator. Early genetic studies in which lux regulatory functions were deleted by transposon insertion mutagenesis demonstrated that the luxR and luxI genes were both required for the proper induction of bioluminescence (Engebrecht et al. 1983). Insertions in luxR resulted in loss of a function which could not be recovered by the addition of autoinducer, whereas insertions in the luxI gene were complemented by autoinducer addition. These observations led to a model which suggested that the luxI gene product was required for autoinducer synthesis and that the luxR gene product interacted with the autoinducer molecule to stimulate transcription of operon$_R$. Recent studies supported that model and demonstrated that a 20-base-pair palindrome, the lux operator, located within the control region is also required for autoinducer-dependent stimulation of transcription of operon$_R$ (Devine et al 1989). Several mutations in the luxR gene which encoded variant LuxR proteins with altered autoinducer responses have been isolated in this manner. Demonstration that these mutant proteins can respond to higher concentrations of autoinducer provide the first evidence supporting the direct interaction between LuxR and autoinducer (Shadel et al. 1990).

REFERENCES CITED

The following references to the extent that they provide procedural details supplementary to those set forth herein, are specifically incorporated herein by reference.

1. Adhya, et al. 1971. pp. 743–746, The Role of Gene S. In, P. R. Hershey, (ed.), The Bacteriophage Lambda. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

2. Baldwin et al. 1989. *J. Biolumin. Chemilumin.* 4:326–341.

3. Bedwell and Nomura. 1986. *Mol. Gen. Genet.* 204:17–23.

4. Bienkowska-Szewczyk, et al. 1981. *Mol. Gen. Genet.* 184:1112–1114.

5. Coleman, et al. 1983. *J. Bacteriol.* 153:1098–1110.

6. Devine, et al. 1988. *Biochemistry* 27:837–842.

7. Devine, et al. 1989. *Proc. Natl. Acad. Sci USA* 86:5688–5692.

8. Dunlap and Greenberg. 1985. *J. Bacteriol.* 164:45–50.

9. Dunlap and Greenberg. 1988. *J. Bacteriol.* 170:4040–4046.

10. Eberhard. 1972. *Bacteriol.* 109:1101–1105.

11. Eberhard, et al. 1981. *Biochemistry* 20:2444–2449.

12. Eberhard, et al. 1986. *Arch. Microbiol.* 146:35–40.

13. Engebrecht, et al. 1983. Cell 32: 773–781.

14. Engebrecht and Silverman. 1984. *Proc. Natl. Acad. Sci. USA* 81:4154–4158.

15. Engebrecht and Silverman. 1986. Regulation of expression of bacterial genes for bioluminescence, p. 31–44, In J. K. Setlow and A. Hollaender (eds.), *Genetic Engineering*, vol. 8. Plenum Publishing Corp., New York.

16. Engebrecht and Silverman. 1987. *Nucleic Acids Res.* 15:10455–10467.

17. Goldberg and Howe. 1969. *Virology* 38:200–202.

18. Hastings and Weber. 1963. *J. Opt. Soc. Am.* 53:1410–1415.

19. Henikoff et al 1990. *Methods Enzymol* 183:111–132.

20. Humphreys, et al. 1976. *Mol. Gen. Genet.* 145:101–108.

21. Joachimiak, et al. 1983. *Proc. Natl. Acad. Sci. USA* 80:668–672.

22. Kaplan, et al. 1985. *J. Labelled Compd. Radiopharm.* 22:387–395.

23. Kaplan and Greenberg. 1985. *J. Bacteriol.* 163:1210–1214.

24. Kaplan and Greenberg. 1987. *Proc. Natl. Acad. Sci. USA* 84:6639–6643.

25. Konisky. 1982. *Ann. Rev. Microbiol.* 36:125–144.

26. Kunkel, et al. 1987. *Methods Enzymol.* 154:1367–1382.

27. Lanzer and Bujard. 1988. *Proc. Natl. Acad. Sci. USA* 85:8973–8977.

28. Maniatis, et al. 1982. *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

29. Meighen. 1988. *Ann. Rev. Microbiol.* 42:151–176.

30. Michaelis and Beckwith. 1982. *Ann. Rev. Microbiol.* 36:435.

31. Raab, et al. 1986. *J. Bacteriol.* 167:1035–1041.

32. Rosenberg, et al. 1981. pp. 132–164. In, J. G. Chirikjian (ed.), Gene Amplification and Analysis.

33. Shadel, et al. 1990. *J. Bacteriol.* 172:3980–3987.

34. Slock, et al. 1990. *J. Bacteriol.* 172:3974–3979.

35. Tabor and Richardson. 1987. *Proc. Natl. Acad. Sci. USA* 84:4767–4771.

36. Ulitzur. 1989. *J. Biolumin. Chemilumin.* 4:317–325.

37. Ulitzer and Kuhn. 1988. *J. Biolumin. Chemilumin.* 2:81–93.

The present invention has been described in terms of particular embodiments found or proposed to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of

the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention.

For example, those of skill in the art will appreciate that it is possible to utilize the expression system, vectors and methods of the present invention to express genes whose products vary in their degree of toxicity to the cells in which they reside. Additionally, the regulatory circuitry that is the subject of this invention may be used directly to regulate gene expression or it may be used in an indirect manner by serving as a controlling element in combination with one or more secondary regulatory systems. In a similar manner, the expression systems, vectors and methods of the present invention may rely on induction of gene expression by addition of exogenously added inducer or may rely on endogenous autoinducer. All such modifications are intended to be included within the scope of the appended claims.

What is claimed is:

1. An expression system comprising a lux regulatory circuit, said regulatory circuit further comprising a luxR gene and truncated luxI gene of Vibrio, operably linked to and capable of stringently regulating expression of a gene, a product of which said gene is deleterious to an *Escherichia coil* host cell in which said expression system and said gene are resident.

2. The expression system of claim 1 wherein the lux regulatory circuit further comprises a luxR gene derived from the leftward operon of said lux regulatory circuit, said luxR being operably linked to a control region of said lux regulatory circuit, said control region being operably linked to a portion of a rightward operon, said rightward operon retaining a truncated luxI gene.

3. The expression system of claim 1 wherein said deleterious gene product is the product of a gene which, when expressed, is lethal to said host cell in which said expression system and said gene are resident.

4. The expression system of claim 3 wherein said gene is a lysis gene of bacteriophage λ.

5. The expression system of claim 1 wherein induction of said expression is controlled by addition of an inducer to the environment of said host cells in which said expression system is resident.

6. The expression of claim 5 wherein said inducer is an exogenously added inducer.

7. The expression of claim 5 wherein said inducer is N-(3-oxo-hexanoyl) homoserine lactone.

8. The expression of claim 1 wherein said lux regulatory circuit is operably linked to and capable of exerting control over at least one secondary regulatory system.

9. The expression system of claim 1 wherein the lux regulatory circuit further comprises a luxR gene derived from the leftward operon of said lux regulatory circuit, said luxR gene being operably linked to a control region of said lux regulatory circuit, said control region being operably linked to a portion of a rightward operon, said rightward operon comprising a promoter and a truncated luxI gene.

10. A vector comprising a lux regulatory circuit, said regulatory circuit further comprising a luxR gene and truncated luxI gene of Vibrio, operably linked to and capable of stringently regulating expression of a gene, a product of which said gene is deleterious to an *Escherichia coli* host cell in which said lux regulatory circuit and said gene are resident.

11. The vector of claim 10 wherein the lux regulatory circuit further comprises a luxR gene derived from the leftward operon of said lux regulatory circuit, said luxR being operably linked to a control region of said lux regulatory circuit, said control region being operably linked to a portion of a rightward operon, said rightward operon retaining a truncated luxI gene.

12. The vector of claim 11 wherein said truncated luxI gene is operably linked downstream of its truncated portion to a multiple cloning site.

13. The vector of claim 11 wherein the vector is pGS103.

14. A method for expression of a deleterious gene comprising:

(a) constructing a vector which is comprised of a lux regulatory circuit, said regulatory circuit further comprising a luxR gene and truncated luxI gene of Vibrio, capable of being operably linked to and capable of stringently regulating expression of said deleterious gene;

(b) operably linking said deleterious gene to said lux regulatory circuit;

(c) inserting said vector containing said lux regulatory circuit operably linked to said deleterious gene into a suitable *Escherichia coli* host cell;

(d) allowing multiplication and growth of said host cell to a suitable level; and,

(e) inducing expression of said deleterious gene by introduction of an exogenous inducer into the environment of said host cell.

15. The method of claim 14 wherein said deleterious gene produces a product which, when expressed, is lethal to said host cell.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. :5,196.318

DATED : March 23, 1993

INVENTOR(S) : Thomas O. Baldwin, et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 19:

In claim 1, line 6, please delete "*coil*" and insert therefor --*coli*--.

In claim 6, line 1, please insert --system-- after "expression".

In claim 7, line 1, please insert --system-- after "expression".

Column 20:

In claim 8, line 1, please insert --system-- after "expression".

Signed and Sealed this

Thirtieth Day of November, 1993

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks